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Key Leaders' Opinion on MicroRNA and Myocardial Infarction

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Key Leaders' Opinion on MicroRNA and Myocardial Infarction (FIRST EDITION)

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Will scholarly journals perish?

Will scholarly journals perish? This is a question that has puzzled me for years.

The introduction of online journals has resulted in the inevitable recession of print journals. The uprise of the open access journals has been changing the structure of scholarly journals ceaselessly. What keeps turning over in my mind is the open access of clinical trials data. What would the bigger picture be if open access to clinical trials data became the mainstream?

It is interesting that with the primary bottleneck being the availability of open data, the Big-data Clinical Trial (BCT) seems to stay where it was in spite of the increasingly popularity of "Big Data" among scientists. It has due to the fact that the fact that without open data, a statistical analysis is restricted to a particular area (or several areas). Even with big enough data, the study can only be termed as "research with big data sets" rather than "big data research", which are totally different concepts. Big Data is constituted by a plurality of dimensions. On one hand, for an individual (e.g., a patient), the relevant data covering his/her disease course is large enough; on the other hand, for the entire population, as more as individuals (e.g., patients) are expected to be included, to contain all the elements just like the "universe set" in set theory; by doing so, scientists expect to carry out the so-called clinical studies in real-world settings.

Why do real-world-based clinical trials so appealing? It is understandable that there is a likelihood that the results and conclusions will be altered in studies targeting the same issue using the same research method with the sample size changed. Indeed, the probability of such a likelihood is quite high. In many top journals, it is a common phenomenon that some authors tend to validate the results of one study in another population using the same research method. However, if the results are "validated" in one population, it only means that they are "repeatable". Will the results also be repeatable in the second, third, and populations beyond? If the attempts are not extensively iterated as they should be, this "validation" is, in a sense, equivalent to self-deception.

When clinical research data is open accessed, we can easily integrate data from multiple centers for statistical analysis and meanwhile "validate" the results in multiple populations. If this is the case, then another question arises: can everyone easily publish his/her results/papers in high-profile journals such as the *New England Journal of Medicine*? My answer is NO.

When the open access to clinical research data becomes mainstream, it will be easy to have access to a constantly updated database on the Internet. Simply by clicking on a button, we could obtain the statistical results of the most current data. Another click would display the validation results based on a specific population. The database would be updated at regular intervals (e.g., 1 month or 1 day), and the statistical results would likely also be changed accordingly. At that time, the question may change to "would any researchers publish their findings in a journal?" Well, even if someone is still keen to write such articles, journals may be reluctant to publish them because of the indefiniteness of the findings with the risk of being overturned at anytime.

Eventually here it comes the serious question: will scholarly journals perish? My answer is still NO. In what way then can scholarly journals continue to be relevant?

During my Business Administration course, my teacher distributed to us an article from the Case Study column of the *Harvard Business Review*. In this highly respected journal, articles in this column often present one case first, followed by the comments from two experts. These comments could either support or oppose each other. My teacher asked us to study the case, read through the comments and then form our own point of view on the case. He encouraged us to interpret the case from different perspectives independently, which I found to be a fairly practical exercise.

Thinking of this course brought up a possible answer to me. When the open access to clinical research data becomes mainstream, the entire publishing industry, especially the publication of "scholarly journals", would eventually experience revolutionary change. It may no longer focus on rigid and cold outcomes but would surely shift focus towards the reflection on problems, the update of insights, and the integration of science and arts.

AME Medical Review Series is a product born of this new mentality. As an attempt, we decided to invite international experts to provide their views on a specific topic to share their insights with more clinicians with the aim that this will ultimately benefit more patients. The first chosen topic for the series is the currently controversial one: conventional surgery versus

stereotactic body radiotherapy for the early stage lung cancer. As the first book to the series, we hope it will give you a glance of the coming changes.

The book series will be written by a group of individual experts who are willing to contribute medical reviews and comments for those readers who are specially interested in clinical research and medical reviews. While the book in your hand may be about a difficult subject, we do hope we have presented it in an accessible manner. We would be overjoyed if it can, in any way, bring you thought and inspiration.

Stephen D. Wang
Founder and CEO,
AME Publishing Company

In recent years, research on new types of myocardial infarction markers has been supported by the Ministry of Science and Technology, the National Natural Science Foundation of China, and the Chinese Academy of Sciences. Yang and colleagues have made remarkable effort in this field. The editorial team of this book has been committed to reporting on the discoveries made by the research on new markers of myocardial infarction. Moreover, in the past few years, it has also shared a number of team research results in various academic journals.

As human beings continue to understand the laws of life, the focus and fashions of scientific research are constantly changing. Recently, the field of life sciences has undergone tremendous change, from Genomics to Proteomics to RNomics. The study of non-coding RNA represented by microRNA has become a hotspot for research and an expanding frontier in the life science disciplines of cell biology, developmental biology, neurobiology, and molecular immunology among others. The editorial member of this book have conducted an in-depth exploration of miRNAs in plasma samples of patients with acute myocardial infarction. This book contains all the research results to date, which include the relationship between acute myocardial infarction and miRNA, the function and mechanism of common miRNAs related to myocardial infarction, such as miRNA-1, 138, 22, 197, 233, etc.and research findings and remarks by researchers at home and abroad in many related fields.

Currently, there are still many gaps in our understanding of the study of new myocardial infarction markers. The purpose of this book is to illuminate more of this uncertainty, and to inspire peers engaged in their own research on new types of myocardial infarction markers. It is hoped that it can act as a great guiding influence for medical workers, and it play a significant role in the continuing research of new myocardial infarction markers, the analysis of microRNA in patients with acute myocardial infarction, and the improvement of diagnosis and treatment of a AMI, while promoting the further development of research in related fields both here and abroad.

The editorial members and the research team should continue to accumulate and update the evidence in this area, provide feedback to the reader, so as to offer readers a broader platform for the development of new markers of myocardial infarction. From reading this book, I have experienced the enterprising spirit which has strived for the continued discovery of new markers of acute myocardial infarction. Accordingly, I am very gratified to write this foreword and pleased to offer congratulations in completing the publication of *Key Leaders'Opinion on MicroRNA and Myocardial Infarction*.

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miRNAs in CVD and myocardial infarction

Over the last century, cardiovascular diseases (CVD) like myocardial infarction (MI) and stroke have overtaken infectious diseases as the leading cause of mortality worldwide. Researchers have put tremendous efforts into clinical and epidemiological studies, enabling us to unravel large parts of the etiology of the underlying risk factors leading to cardiovascular pathologies. These factors include ageing, arterial hypertension, diabetes, hyperlipidaemia, obesity and tobacco smoking, all of which result in atherosclerosis that becomes the main trigger for ischemic forms of CVD like MI. Despite some crucial revelations, CVD will likely remain the number one reason of death in the foreseeable future, while our current knowledge even predicts an exacerbation in the prevalence of CVD. Thus, in-depth investigations into key determining molecular and epigenetic mechanisms have been initiated, hopefully enabling us to discover novel and powerful markers of disease as well as potential therapies. Recent advances in the field have enabled us to unravel and functionally assess the role of non-coding RNAs (ncRNAs) in disease development and progression.

Insights into the transcriptomic regulation of different species were able to show that approximately 80% of our genome is getting transcribed. However, only 1–2% of this gets translated into protein, generating a large number of ncRNA transcripts. This ncRNA was long believed to be a sequencing artefact or simply non-functional 'junk DNA'. Over the past 10-15 years however, it has been indicated that ncRNA are key mediators and 'fine-tuners' in gene expression and epigenetic control, in particular in different stages of disease development and progression. We are still only scratching the surface of understanding the different forms and subclasses of ncRNAs, and the regulatory function they play in the homeostasis of an organism. These ncRNA subspecies include small nuclear and small nucleolar RNAs, microRNAs (miRNAs), Piwi-interacting RNAs, Y-RNAs and the extensive group of long-noncoding RNAs, which include long-intergenic-noncoding RNAs and natural antisense transcripts. Another interesting form of ncRNAs are circular RNA, which appear to be promising biomarkers due to their stable expression and extended protection from degradation within the circulation.

Currently, miRNAs appear as the most extensively studied and best characterized ncRNA subgroup. miRNAs are well-conserved and upon maturation in the cytoplasm 18-22 nucleotides short transcripts that can act as key post-transcriptional regulators of gene expression not only in humans, but also plants, nematodes, yeast, and other animals. By now, miRNAs have been identified to play major roles in almost every biological process via mediating the translation of target messenger RNAs (mRNAs) and thus their stability.

This present book entitled "*Key Leaders' Opinion on MicroRNA and Myocardial Infarction*" focuses mainly on the *in vivo* role of miRNAs in the pathogenesis of developing and existing myocardial disease. The therapeutic potential of targeting miRNAs and their contribution as biomarkers in CVD pathologies is thoroughly presented. Numerous preclinical experimental CVD models have indicated that either inhibition or induction/overexpression of a single miRNA can augment or diminish CVD development and/or progression.

miRNA modulation can be powerfully enforced by utilizing so called antimiRs or antagomiRs (synthetic antisense oligonucleotides) that bind and silence miRNA expression, or by using miRNA mimics or pre-miRNAs that act similarly to endogenous miRNAs. Human and animal efficacy data exists that implies the promising role antimiRs might play in disease management. Important features include the frequent conservation of target miRNAs across species and the small molecule size. Several candidate miRNA inhibitors have shown that they can silence their putative miRNA with convincing affinity and specificity. Intriguingly, until now no immunogenic or toxicity issues were reported in human Phase I and II clinical trials. One disadvantage of targeting miRNAs in CVD might be their ubiquitous expression throughout organs and different tissues of the human body. This favors local and cell type specific tools for miRNA modulator delivery, which would limit undesired off-target effects in organ systems in which anti-miRNAs can accumulate to a much higher extent than the targeted cardiovascular system. It will be interesting to see the results of the first miRNA-based therapy trials in CVD. Several candidates exist, with anti-miR-92a in patients with myocardial ischemia being the current frontrunner for a CVD-miRNA-therapy trial in humans. First-in-patient studies and subsequent data is here expected for 2019.

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Despite the past development of drugs, i.e. statins, that efficiently lower plasma cholesterol levels and thereby reduce the chance of developing atherosclerotic lesions, the risk to die from a cardiovascular event remains very high. More specifically, death of myocardial infarction is still the predominant cause of death worldwide. As such, there is clear need for the development of alternative cardiovascular therapies. In this light, it is of interest to see that many associations have been found between differences in the cellular expression and/or plasma level of a specific class of small non-coding RNA molecules, the ~22 nucleotide long so-called microRNAs, and the occurrence of myocardial infarction. MicroRNAs through binding to their target transcripts negatively impact on the gene expression of a wide variety of proteins, and - in this way - are able to modulate many processes involved in the development of atherosclerotic lesions, the de-stabilization of these lesions, and the generation and recovery from a myocardial infarction. Notably, microRNAs constitute valuable drug targets as they can be readily administered to humans. Furthermore, their activity can also be easily diminished through administration of antagonizing nucleotides, i.e. antagomirs. When taking the aforementioned findings into account, one can foresee a bright future for microRNAs in cardiovascular drug development. Providing insight in the (causal) contribution of microRNAs to cardiovascular disease pathology, i.e. through basic proof-of-principle and clinical studies, will hopefully aid in a speedy development of novel microRNA-based cardiovascular therapies.

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"*MicroRNAs have it all*".

This book is the first edition of a series of scientific papers from Key Opinion Leaders' in the field of microRNAs and myocardial infarction. The reader will be guided through an extensive review of the available literature related to the role of microRNAs in myocardial infarction.

MicroRNAs (miRNAs) are small single-stranded RNA molecules that regulate target gene (messenger RNA) expression, either through inhibition of translation or activation of degradation. More than 2,500 mature human miRNAs have been characterized so far. They are ubiquitously expressed and are involved in virtually all pathophysiological processes.

In the heart, miRNAs have been extensively described. Some of them have been shown to be enriched in skeletal muscle, the so-called myomiRs (miR-1/133a-b/206/208a-b/486/499). These miRNAs have attracted a lot of attention for their potential to diagnose myocardial infarction or to aid in fighting heart failure. Since their discovery in the circulation (1), miRNAs have been the topic of a plethora of studies, which have been facilitated by the development and commercialization of multiple kits and molecular biology tools. These tools allowed to measure circulating miRNAs and manipulate their expression levels in cells, thereby allowing to test both their biomarker value and their therapeutic potential. Hence, miRNAs have appeared as promising candidates for Theranostics*, with some potential to advance the development of personalized and precision medicine. In the context of myocardial infarction, miRNAs have the potential to aid implementing personalized healthcare since they have some diagnostic and prognostic value and are involved in the development of heart failure that sometimes occurs after myocardial infarction (2).

However, much remains to be done for a complete knowledge of the role of miRNAs in atherosclerosis, plaque rupture, left ventricular remodeling, heart failure (...). Cost-effective molecular diagnostic kits have to be developed to allow a reliable measurement of circulating miRNAs. The therapeutic value of novel strategies to regulate the expression levels of miRNAs has to be tested.

This book gathers essential information on the emerging role of miRNAs in myocardial infarction, from biomarker to functional and translational studies. It provides directions for future research and reveals challenges that remain to be addressed before miRNAs can find their practical application for the benefit of myocardial infarction patients (3).

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* Theranostics is a new biomedical field where targeted diagnostic tests are combined with specific targeted therapy.

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Myocardial infarction is pathologically defined as myocardial cell death due to prolonged ischemia, which is the most severe manifestation of coronary artery disease. A sudden rupture of plaque and formation of thrombus leads to acute myocardial infarction. Once the oxygen supply is occluded, the onset of myocardial infarction is initiated as little as 20 min after and the complete myocardial cell necrosis happens in a few hours. Prolonged ischemia leads to the loss of heart contractility due to the poor proliferation capability of the myocardial cell. The timely revascularization of the occluded artery is the key for myocardial infarction therapy. However, these treatments only reduce the severity of coronary artery disease, rather than restoration of the contractility of the infarcted heart. Therefore, novel therapeutic strategies to reduce the myocardial cell death and/or stimulate heart regeneration are highly desirable for the future.

Cardiac cell death plays a critical role in the pathogenesis of myocardial infarction, due to the terminal differentiation and loss of regenerative ability of cardiomyocytes. Myocardial infarction usually involves three main types of cell death process including apoptosis, necrosis, and autophagy. MicroRNAs are defined as single-stranded non-coding RNAs with around 22 nucleotides. Cardiac cell death processes are regulated by a variety of molecules, among which microRNAs have shown outstanding regulatory functions. Recently, a large body of research has emphasized the importance of microRNAs in regulating apoptosis, necrosis, and autophagy in cardiomyocytes, which play a decisive role in myocardial infarction. Nevertheless, more regulatory pathways and the link between different pathways of microRNAs regulating cardiac cell death need to be clarified urgently.

The significance of microRNAs in regulating myocardial infarction has been well emphasized by multiple studies. Several microRNAs are downregulated/upregulated depending on the type of myocardial injury. The significant changes in their expression pattern upon myocardial infarction highlights their contribution in regulation of pathogenesis of myocardial infarction.

The regenerative capacity largely declines within seven days after birth and remains very low in the adult heart. Thus, the activation of endogenous heart regeneration and the triggering of cardiomyocytes renewal could provide new clues for the therapy to treat myocardial infarction. Different approaches have been proposed to regenerate new cardiomyocytes: (I) to promote resident cardiomyocytes proliferation by inducing them to re-enter the cell cycle; (II) to activate endogenous stem cells or progenitors such as cardiac stem cells differentiation; (III) to stimulate endogenous regeneration through direct reprogramming from cardiac fibroblasts into cardiomyocytes. It has been demonstrated that microRNAs are critical regulators of these processes and exhibit as potential new therapeutic targets for myocardial infarction.

Circulating microRNAs in the blood have recently emerged as potential biomarkers for the diagnosis or prognosis of myocardial infarction due to their stability and specificity in plasma. A large body of studies explored the fact that microRNAs are leaked from the heart into the circulation after myocardial injury, during which their expression is elevated and dynamic. Circulating microRNAs are stable and can be easily quantified by real-time PCR assay. Among these abundant microRNAs in the heart, four cardiac-enriched microRNAs (miR-208, miR-499, miR-1, and miR-133) are consistently found to be increased in the plasma of acute myocardial infarction patients. Although the potential value of microRNAs as biomarkers has been established in small-scale studies, it is difficult to validate them in large cohorts of patients with myocardial infarction. In addition, the methods of microRNAs detection need to be optimized. The standardized assays for the detection of microRNAs in patients may reduce the inconsistency and microRNAs may become potential biomarkers for diagnosis of myocardial infarction patients. More prospective studies are underway to assess the diagnostic value of microRNAs as biomarkers. The sensitivity and specificity of circulating microRNAs have an attractive prognostic value in response to myocardial infarction, and relevant research is being designed and carried out.

Accumulating evidence reveals that microRNAs function as pro- or anti-myocardial infarction factors through their influence on myocardial cell death and cardiomyocyte regeneration pathways. So far, significant progress has been made to unveil the microRNAs-regulated signaling pathways of myocardial infarction, which has improved our understanding of heart pathogenesis.

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An increasing number of studies have recently demonstrated that the human genome is dynamically transcribed and leads to the production of a complex world of RNAs. Of those, there is a wide spectrum of RNAs that do not encode proteins, named as non-coding RNAs (microRNAs, miRNAs), whose role remained unclear for many years. Evidence suggests that miRNAs are associated with human disease. Recent studies indicate that altered expression and function of miRNAs is strongly associated with cardiovascular disease including arrhythmias, hypertrophy and myocardial infarction.

Several methods have been developed to study the expression and quantification of miRNAs, but each individually still has practical issues and technical limitations which need to overcome in order to adapt the use of miRNAs in routine clinical practice. Despite the recent advances and promising results associating miRNAs with myocardial infarction, there is still a large proportion of studies that failed to show superiority of miRNAs over classic biomarkers. miRNAs could be of value as adjunctive and combinative biological tools, focusing particularly on the early diagnosis of acute myocardial infarction and to distinguish unstable angina from other non-cardiac causes of chest pain. However, there is need for more clinical studies to evaluate the role of miRNAs in myocardial infarction as stable and sensitive biomarkers.

In the present book, we highlight the potential role of miRNAs as diagnostic tools as well as possible therapeutic targets in cardiovascular disease and more specifically in myocardial infarction. We hope that this book will trigger further research on the role of miRNAs in this field.

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Over the past decades cardiac biomarkers have become a cornerstone in the diagnosis of acute myocardial infarction (AMI) and cardiac injury of other causes, and more recently of heart failure as well. We are beginning an era when it may be possible for biomarkers to direct treatment to optimize patient care, which must be the final goal with all routinely used cardiac biomarkers. A currently very popular research topic is circulating plasma micro ribonucleic acid (miRNA) testing. This book excellently compiles the current knowledge on miRNA testing in patients with AMI and other cardiac diseases. miRNA have been first described in the early 1990s, they are small (typically less than 25 nucleotides), single-stranded, endogenous, non-coding RNAs that post-transcriptionally regulate gene expression by destabilizing messenger RNA (mRNA) or translation repression, thereby preventing proteins synthesis. More than 1,000 miRNAs have been identified in the human genome so far. Eventually miRNAs are also secreted from cells into blood, but the biological function of circulating miRNAs still remains to be established. Nonetheless, circulating miRNAs are interesting and attractive candidates for routine laboratory applications, and panels of miRNAs could provide clinical useful information for AMI diagnosis, risk stratification and treatment monitoring. Thus, miRNAs have already been evaluated as diagnostic and prognostic biomarkers in a variety of human diseases, in particular cancer or cardiovascular disorders, which yielded numerous associations between miRNAs and different types of diseases. Although examples exist where deregulated expression of a single miRNA is indicative for a disorder, the simultaneous analysis of the expression of multiple miRNA usually provides more accurate information. But the interpretation of large panel test results may be challenging requiring expert knowledge in bioinformatics, which clearly is a limitation for routine use.

The next steps for making the way of miRNA testing from research to routine use in cardiovascular diseases will be to replicate and confirm promising available clinical data of the discovery phase in large-scaled, ideally multi-centre clinical trials using a small panel of candidate miRNAs with appropriate pre-analytics and analytics in a clinically relevant patient and control population as validation. Analytical issues still need to be overcome before routine use and explain the poor replicability of circulating miRNA studies. Currently miRNA testing is still tricky, time consuming and purification steps, methods, and normalization remain to be harmonized or standardized and analytical steps automatized. These analytical issues make it very difficult to compare the sample and method dependent published clinical study results. Quantitative reverse transcription PCR (qRT-PCR) still is the most widely used method, which precludes heparin or citrate as an anticoagulants of blood samples as both can inhibit PCR amplification. In addition, although circulating miRNAs are remarkably stable molecules, e.g.hemolysis during blood sampling has to be avoided and whole blood must be processed immediately for platelet free plasma to avoid in-vitro miRNA release from blood cells. Alternatively specific collection tubes in which cells of whole blood samples are directly lysed and miRNA expression is thereby stopped are available. However, a high biological variation between individuals appears to be an issue as well.

Finally, circulating miRNAs have emerged as novel biomarkers in cardiovascular diseases. But, as with other heavily investigated novel biomarkers, the coming years will show whether miRNA testing will make the way from research to routine use. This book is an excellent companion for all interested in the field of miRNA testing in cardiovascular diseases.

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Acute myocardial infarction is one of the leading causes of mortality and morbidity worldwide. On the basis of literature it was defined as myocardial cell death due to prolonged ischemia. In the last two decades the progressive developments in the field of mechanical (percuteneous coronary intervention) and pharmacological therapies have contributed to reduce myocardial damage and subsequent remodeling. Several studies published in last years, have revealed the diagnostic value of circulating microRNAs for acute myocardial infarction detection. MicroRNAs are a class of single stranged and non coding RNAs which are involved in some cardiac disorders such as myocardial infarction, cardiomyocyte hypertrophy and, also, in hear failure. However, the diagnostic capacity of miRNAs for acute myocardial infarction is still controversial; some studies demonstrated that microRNAs are involved in regulating cardiac cell death and regeneration after myocardial infarction. Infact a growing number of studies showed the role of microRNAs in regulating apoptosis, necroptosis and autophagy in cardiomyocytes. Some microRNAS such as miR–17, miR–18a, miR–19a, miR–19b has been demostrnated to be associated with cardiomyocytes proliferation; miR–548–3p, miR–509 3p hav been demonstrated to be involved in regulation of cell cycle progression. These are only some correlations recently demonstrated. Furthermore an interesting alternative to compensate the cardiomyocytes loss during acute myocardial infarction it could be to stimulate proliferation and differentiation of cardiac stem cells or their progenitors. Some studies *in vitro* showed that overexpression of miR – 1 family enhances cardiac differentiation of cardiac progenitor cells. On the basis of the date we can find in the literature, microRNAs hava a great potential to offer a novel potential diagnostic biomarkers in the setting of acute coronary disease and acute myocardial infarction. We expect more large scale, multicenter clinical studies and trials to validate the knowing data of specificity and sensitivity of microRNAS. This volume reported and summarize some recent review and studies about microRNAs and acute myocardial infarction and I hope it can answer the doubts.

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A recent surge in the exploring noncoding RNAs in stem cell research has ignited a field of discovery into many diseases including heart diseases. Heart diseases continue to be among the leading causes of death in Western countries. The most common heart diseases such as Myocardial infarction, atherosclerosis and hypertension which are mainly affect the heart, lungs and the blood vessels. Several independent risk factors have been identified and shown to be responsible for cardiovascular diseases. Although important progress has been made in the treatment of heart failure during the last decade, most interventions relieve symptoms or prevent disease progression. Thus, an improved knowledge and treatment or curative regimen is desperately needed. Recently, scientists have demonstrated that RNA functions not only as an intermediate molecule between DNA and protein, but also plays a critical role in regulating gene expression. Some of the RNAs are functional in cells but do not encode proteins. Hence, these RNAs are called noncoding RNAs (ncRNAs). Approximately 10% of the host genome consist of ncRNAs and are occupied at intergenic or intragenic regions. These ncRNAs play an important role in regulating genes that are involved in controlling the transcriptional or translational pathways. Importantly, ncRNAs are having diverse biological functions like development, differentiation, growth, and metabolism. Among ncRNAs, the short interfering RNAs and microRNAs (miRNAs) have been extensively studied, but their specific functions yet to be identified. In recent years, miRNAs are efficiently studied as one of the important candidates for involvement in most biological processes and have been implicated in many human diseases. Thus, the identification and the respective targets of miRNAs may provide novel molecular insight and new therapeutic strategies to treat diseases.

Number of recent studies has shown that miRNAs are essential for the normal development and physiology of various organs, including the heart. Studies have also started to characterize the link between miRNAs and different aspects of cardiac pathogenesis as well as proliferation, differentiation, function and maintenance of cardiac cells. Moreover, congenital heart anomalies can be associated with the dysregulation of specific miRNAs. The recently developed high-throughput approaches revealed the miRNA size, and their target, and the connectivity of the miRNA-dependent regulatory network. One step further, the expression levels of miRNAs and their decay rates have been identified in individual cell types. These works together help us to understand miRNA-dependent gene regulation to study the response of the entire network. During the past decade, numerous research articles have shown a wide knowledge about the basic mechanisms of miRNAs, biogenesis and its functions in the circulatory system. Although miRNAs are richly expressed in the heart, relatively little is explored about the multi-functional effect of these molecules in the heart.

Circulating miRNAs in patients with MI have been examined very recently. It was reported that miRNAs served as a critical modulating regulator, which participates in almost all aspects of cardiovascular diseases and vascular biology. Studies demonstrated that miRNAs also existed in blood, in the form of circulating miRNAs and are resistant to endogenous ribonuclease activity and can be present in a remarkably stable form during pathological conditions. More importantly, circulating miRNAs are currently explored as biomarkers in a wide range of cardiovascular conditions, including atherosclerotic disease. Based on genome-wide studies, thousands of miRNAs exist, however as of now only a limited number of functional miRNAs have been sequenced, identified and characterized. It takes part actively in regulating splicing, localization, stability, and translation of the target mRNAs. MicroRNAs that are capable of interfering with either complete or partial complementary to the cellular mRNAs, would be a useful treatment strategy for various diseases. I hope that the literature and examples provided here will illustrate the diversity of mechanisms regulating miRNAs while protecting the young and old heart. Certainly, many more needs to be discovered and the full potential of miRNA as therapeutic agents to be revealed. Thus, miRNAs have a promising and an exciting future in the field of research.

In this book, studies have highlighted the mechanisms by which miRNAs regulate various biological functions in model systems and could be a potential molecular therapeutic target for various heart diseases. Moreover, the authors discussed the functional roles of miRNAs and its potential use of diagnostic biomarker for cardiovascular diseases, as well as the limitations and challenges in miRNA-based therapy.

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Which future for circulating microRNAs as biomarkers of acute myocardial infarction?

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Provenance: This is a Guest Viewpoint commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Wang KJ, Zhao X, Liu YZ, *et al.* Circulating MiR-19b-3p, MiR-134-5p and MiR-186-5p are Promising Novel Biomarkers for Early Diagnosis of Acute Myocardial Infarction. Cell Physiol Biochem 2016;38:1015-29.

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Cardiovascular disease remains the first cause of death and disability around the world according to the World Health Organization (http://www.who.int/cardiovascular_diseases). An estimated 31% of deaths worldwide are due to cardiovascular disease and, in many countries, cardiovascular disease is responsible for more than twice as many deaths as cancer (1). Coronary heart disease and stroke are especially devastating and, among coronary heart disease, acute myocardial infarction (AMI), known as "heart attack", is the most common and the most deadly condition. Obstruction of a coronary artery leads to AMI and to the necrosis of a part of the heart due to rupture of blood supply. An early diagnosis of AMI in patients presenting with chest pain is necessary to rapidly restore blood flow to the heart to limit the extent of myocardial necrosis, which largely impacts patient outcome. Currently, AMI diagnosis is based on electrocardiogram findings and measurements of blood biomarkers of myocardial damage, among which cardiac troponins (cTns) are the most widely used. High-sensitivity troponin assays have been developed, but they suffer from a lack of specificity since elevation of cTn levels can be due to non-cardiac causes. Therefore, there is an unmet need for novel, early and specific biomarkers of AMI.

In the early 2000's, a new class of RNA molecules called microRNAs (miRNAs) emerged. miRNAs are small 20–22 nucleotides-long single-stranded non-coding RNAs able to down-regulate the expression of protein-coding genes, either through inhibition of the translation of target messenger RNAs or induction of their degradation (2). In the heart, miRNAs are widely expressed and regulate multiple physiological and pathological pathways such as apoptosis, fibrosis or angiogenesis (2).

The discovery by Mitchell and co-workers that miRNAs are present and stable in the bloodstream (3) triggered a wealth of investigations of their biomarker potential. Of note, circulating miRNAs can be either released by dying cells or be actively secreted by living cells, acting as paracrine factors. The former possibility led to the hypothesis that circulating miRNAs emanating from dying cardiomyocytes after AMI might constitute a novel class of biomarkers of AMI. This hypothesis was tested by multiple groups and led to the publication of many reports since 2010 [reviewed in (2)]. From animal studies and small-scale studies conducted in humans, it appeared that many miRNAs are indeed released from dying cardiomyocytes after AMI. In patients with hypertrophic obstructive cardiomyopathy, circulating levels of muscle-enriched miR-1 and miR-133a were significantly increased 15 mins after transcoronary ablation of septal hypertrophy (4), supporting the hypothesis that heart-derived miRNAs may constitute early diagnostic biomarkers of AMI. The excitement around the diagnostic potential of miRNAs for AMI was tempered by large-scale studies in AMI patients and patients with chest pain reporting that circulating

miRNAs fail to provide an incremental diagnostic value over traditional markers including cTns (2,5). This disappointing result was nevertheless limited by the fact that patients in these retrospective studies were initially diagnosed with cTns. Additional prospective studies might bring back some hope, such as a recent study from Wang and colleagues reporting that plasma levels of miR-19b-3p, miR-134-5p and miR-186-5p reached a peak in the 4 hours after admission for AMI, while cTnI showed a peak only after 8 hours (6). All three miRNAs had a robust diagnostic capacity and a 3-miRNA panel discriminated AMI patients from controls with an area under the curve close to 0.90 at admission. However, this study is limited by a low sample size (18 AMI and 20 controls) and the absence of multivariable analyses to address the added diagnostic value of miRNAs on top of existing markers.

While the benefit of using circulating miRNAs for the diagnosis of AMI may be limited, mostly due to the accuracy and rapidity of high-sensitivity cTns assays, there might exist a window of opportunity for prognostication purposes. Indeed, predicting outcome after AMI is still a challenging task. The heart failure biomarkers brain natriuretic peptides (BNPs) are poor predictors of the adverse left ventricular remodelling process leading to heart failure, mainly due to fluctuating plasma levels in the few hours following AMI (7). In two independent groups of AMI patients, plasma levels of miR-150 at admission predicted left ventricular remodelling and provided an added prognostic value over a multivariable clinical model (8). A 4-miRNA panel including miR-150 improved outcome prediction in a cohort of 150 AMI patients (9). In a recent case/control study with 198 patients, circulating levels of miR-22 were independent predictors of cardiovascular mortality in patients with systolic heart failure from both ischemic and nonischemic origin (10).

While the use of circulating miRNAs as prognostic biomarkers after AMI holds some promise, more remains to be done before these relatively novel markers can reach clinical application. Candidate miRNAs have to be extensively validated; they have to provide very accurate predictions at an early stage after AMI; they have to be stable enough in the blood to be reliably detected; they have to be insensitive to medications and other confounding factors such as age or sex; and finally their methods of detection have to be critically improved to be applicable in a clinical setting. A point-of-care device integrating miRNA measurements in a multifactorial computational model delivering a diagnosis in a relatively short period of time would constitute an attractive "New concept in patient stratification" (Horizon 2020 Work Program 2016–2017. Health, demographic change and well-being. SC1-PM-02-2017) towards personalized healthcare.

In conclusion, although circulating miRNAs are still attracting some interest as diagnostic biomarkers of AMI, the majority of studies conducted so far concluded that miRNAs will have a hard time outperforming the sensitivity and rapidity of high-sensitivity cTn assays. On the other hand, past and recent studies support the use of circulating miRNAs to aid in risk stratification after MI. Future technological developments are needed to translate these research findings into clinical application. Also, more efforts are required before circulating miRNAs can aid controlling the growing burden of heart failure.

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Footnote

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Micro-RNAs as promising biomarkers in cardiac diseases

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Provenance: This is a Guest Commentary commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, Jiangsu, China).

Comment on: Coskunpinar E, Cakmak HA, Kalkan AK, *et al*. Circulating miR-221-3p as a novel marker for early prediction of acute myocardial infarction. Gene 2016;591:90-6.

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miRNA, emerging and promising biomarkers in cardiology

miRNAs are endogenous, small (21-22 nucleotides), single-stranded, non-coding RNAs recently discovered (1). miRNAs complexed with Ago proteins (RISC complex) regulate gene expression by binding to reverse complementary sequences in their target mRNAs leading to mRNA degradation and/or repression of protein translation (2). Circulating miRNAs can be detected in serum or plasma and have been proposed as potential biomarkers for cardiovascular diseases, with high sensitivity (3).

Indeed, they are involved in multiple cellular functions (proliferation, migration, differentiation…) (4) and are therefore involved in cardiac and vascular development. A dysregulation in their expression has been suggested to be responsible for cardiovascular disease. Major implications were demonstrated in several pathologies as congenital cardiac disease (5), hypertrophy, fibrosis, arrhythmias and atherosclerosis (6).

Expression of miR-378 dramatically decreased as well as expression of miR-133 and miR-1 in mouse models of heart failure and in human tissues (7,8). Concerning cardiac fibrosis, several miRNAs were incriminated as miR-133, miR-21 and miR-29, involved in fibroblasts proliferation, collagen synthesis and Connective tissue growth factor (CTGF) signaling (9). In a rat model, miR-433 was consistently elevated in three various models of heart disease with prominent cardiac fibrosis (10).

The implication of miRNAs in heart failure was also demonstrated, particularly miR-25 whose increased expression can depress cardiac function (11). Interestingly, miRNAs secreted by cardiac fibroblasts act as paracrine mediators of cardiomyocyte hypertrophy (12).

Thereby, miRNAs appear as attractive biomarkers in cardiovascular field, easily assessed, with a robust stability in the plasma and an excellent sensitivity (13). A potential combination of several miRNAs can be considered to improve diagnostic performance.

However, few miRNA are tissue-specific and their plasma level can change depending on the physiological or the pathological situation, independently from the initial disease. Above all, there is a very large number of detectable miRNAs and only a few is likely to provide additional information compared to current validated biomarkers. Their specific interest in clinical settings both for diagnostic, prognostic and even therapeutic approaches remain largely under investigation.

miRNA, promising in myocardial infarction as prognostic, diagnostic and therapeutic tools

In acute myocardial infarction, a rapid diagnostic is necessary to allow an immediate management of patients

Table 1 Opened studies ongoing regarding diagnostic, prognostic or therapeutic effects of miRNA in myocardial infarction

Country	Objectives	Primary endpoint	Number of patient	Estimated primary completion date	NCT
France	(I) To study how the macrophage functions after MI are changed by diabetes (II) To determine the potential role of miRNAs contained in secreted MVs in the transition M1/M2 after MI	Level of expression markers	20	2018	NCT02768935
China	To test the expression of microRNAs related to the syndromes after the intervention of Tongguan capsule	miRNAs spectrum	100	2017	NCT02850627
Spain	To evaluate the prognostic value of circulating miRNAs in patients admitted for STEMI complicated with cardiogenic shock	Mortality	142	2017	NCT02691286
Germany	To develop a biomarker protocol that combines the high sensitivity of cardiac Troponin T and the high specificity of mi-RNA profiles for early and safe identification of non-STEMI in ED patients	Mortality	Unknown	Unknown	NCT02116153
China	To determine whether the expression level of miR-320a are effective as biomarker in evaluating the diagnosis, prognosis and treatment effects of coronary heart disease	Plasma expression of miR-320a in coronary heart disease patients	400	2018	NCT02751060

and ensure a better prognosis, limiting long-term consequences as remodelling and fibrosis leading to chronic heart failure.

Several miRNAs were identified as promising candidates to early detect patients with MI. Among them, miR-1, miR-133a, miR-133b, miR-208a, miR-499, miR-499-5p were advocated but further validation is required (14). A large study evaluated the level of six miRNAs in 1155 patients admitted for acute chest pain. In the 224 patients diagnosed with MI, the levels of miR-208b, miR-499 and miR-320a were significantly higher (15). Thereby, miRNAs as early diagnostic biomarkers in MI seems promising but have to be identified more precisely.

In the context of MI, a potential role of miRNAs as prognostic biomarkers was also raised.

Devaux *et al*. (15) reported that miR-208b predicted survival at 30 days but none of the miRNAs could predict long-term mortality. In a study including 407 patients with a suspected MI, miR-208b and miR-499-5p were identified as potential diagnostic biomarkers of MI with an area under the curve (AUC) around 0.8 and were also predictive factors for outcomes with a prognostic value comparable to cTnT (16).

Taken all these considerations, miRNAs provide promising therapeutic targets. In a pig model of reperfused MI, an intracoronary injection of an anti-miR-92a stimulates angiogenesis and could prevent cardiac remodelling (17). Recently, Gupta *et al*. demonstrated that miR-22, a key regulator of cardiac autophagy, could be an interesting target after myocardial infarction (18). Indeed, they show that miR-22 inhibition post-infarction improved cardiac function and inhibited cardiac remodelling in older mice but not young mice. Thereby, pharmacological inhibition of miR-22 could be promising, especially in older myocardium.

Opened ongoing studies regarding diagnostic, prognostic or therapeutic effects of miRNA in myocardial infarction are presented *Table 1*.

In this paper entitled "*Circulating miR-221-3p as a novel marker for early prediction of acute myocardial infarction*", Coskunpinar *et al*. aimed to identify potential miRNAs to predict early myocardial infarction (MI).

They set 3 objectives to answer:

(I) To compare the serum expression levels of miRNAs 1/in patients with AMI and control subjects with an acute atypical chest pain/dyspnoea and 2/in patients with STEMI and non- STEMI;

(II) To evaluate the potential of these miRNAs to be used as novel diagnostic biomarkers for AMI in patients admitted to emergency department for acute chest pain and/or dyspnoea;

(III) To investigate the relations between the serum levels of miRNAs with the serum levels of previously validated biomarkers, namely troponin I, cardiac risk scores and post-MI left ventricular functions.

In this study, 43 consecutive patients were included, all of them presented acute chest pain and/or dyspnoea, 27 were diagnosed acute myocardial infarction (AMI) and 16 were control subjects. The control subjects presented the same symptoms but without any diagnostic criteria for AMI, particularly no ECG modification and no rise of cardiac enzymes. The blood assessment was performed in all subjects within 4 h of onset of clinical symptoms and miRNAs expression levels were evaluated. Patients with AMI were compared to control patients and within the AMI group, STEMI patients were compared to NSTEMI patients.

The AMI group and control were comparable for baseline characteristics except for left ventricular function. Body mass index (BMI) was the only different baseline characteristics between the STEMI group and NSTEMI group, higher in the NSTEMI group (P=0.026).

The study highlighted three major points:

(I) Nine miRNAs were expressed differently between the control group and the AMI group, without any difference between STEMI and NSTEMI subgroups. Six of these miRNAs were upregulated while the other three were downregulated in patients with AMI. Moreover, the authors highlighted 2 miRNAs which were the most upregulated in the AMI group: miR-4290 and miR-221-3p with a fold regulation of 7.39 and 3.89 respectively. The most downregulated miRNA in this group was the miR-19b-1-5p with a fold regulation of –3.15;

(II) miR221 was significantly positively correlated with Troponin, GRACE and SYNTAX Score while significantly inversely correlated with left ventricular ejection fraction;

(III) miR-221-3p had the better discriminative value for the diagnosis of AMI with a ROC area under curve (AUC) of the level of 0.881 (95% CI: 0.774–0.987; P=0.002), close to AUC for Troponin (AUC: 0.954; 95% CI: 0.892–1.000; P=0.001).

These results are consistent with the upregulation of miR-221-3p in patients with atherosclerosis.

Here, the authors add a practical prognostic information of miRNAs demonstrating an association between the expression levels of mi-RNAs (miR-648, miR-4290, miR-3914, miR-221-3p, miR-127-5p) and cardiac scores as well as cardiac function assessed by echocardiography, in patients with AMI. Among these miRNAs, miR-221-3p had a high discriminative value and significant relations with Troponin, GRACE and SYNTAX score and left ventricular systolic function. Likewise, this biomarker may be useful in daily practice for early prediction of AMI and could provide a prognosis value in this context.

From a pathophysiological point-of-view, miR-221-3p is suggested to facilitate the development of vulnerable coronary plaques, coronary artery atherosclerosis and severe endothelial dysfunction by using molecular pathway such as Netrin/DCC induced pathway.

This study presents three main limitations: first, a very small number of patients were included. Secondarily, miRNAs expression levels were measured only once, no time-course or assessment after treatment to evaluate its effect are available. Importantly, the quantification of miRNA expression in different independent study cohorts was not performed and this will be a crucial step for further clinical development. Finally, it is striking that altogether the studies performed worldwide on plasma miRNA identification post AMI present a relatively poor overlap in the subset of differentially regulated miRNAs. This suggests potential methodological biases in the timing and handling of samples and quantification methods that will require to be solved in the future for efficient and reliable use of miRNAs detection in clinics. Alternatively, but not exclusively to the potential methodological biases, one can hypothesize that these differences in candidate miRNAs for AMI arise from different genetic backgrounds and environmental causes, which will generate a very interesting line of research for personalized medicine, a much awaited promise for the future in clinics.

Nevertheless, results presented in this study are innovative. The authors highlighted the miR-221-3p as a potential biomarker not only for early diagnostic of AMI but also for prognostic evaluation after AMI (because of its association with post-MI left ventricular systolic function). Further investigations are necessary to make clear the links between miRNAs products and pathophysiological mechanisms. Furthermore, larger clinical studies have to confirm the early predictive and the prognostic values of miR-221-3p in the context of MI in the aim to develop adapted therapeutic strategies. Finally, a key point for clinical development in this field of research will be to determine whether any of the identified miRNA can present at least an as high discriminating ROC value than

the validated Troponin I biomarker for AMI diagnostic.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Circulating micro-RNAs as biomarkers of coronary artery disease: is it ready for primetime or still a work in progress?

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Provenance: This is a Guest Editorial commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: O Sullivan JF, Neylon A, McGorrian C, *et al*. miRNA-93-5p and other miRNAs as predictors of coronary artery disease and STEMI. Int J Cardiol 2016;224:310-6.

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MicroRNAs (miRs) are small non-coding RNAs that play a significant role in epigenetic regulation of gene expression (1,2). Several miRs have been established to play crucial roles in a spectrum of different cardiovascular diseases (3,4). Since the initial identification of detectable levels of circulating miRs in plasma (5-7), circulating miRs have become the spotlight of investigation as potential biomarkers for several diseases. Micro-RNAs in circulation have two important features of ideal biomarkers: they are remarkably stable and safe from degradation by RNases, and they are easily measured in the circulation using sensitive and specific quantitative PCR techniques. Thus, circulating miRs can be linked to specific disease processes or to specific tissue and/or cell types.

In a recent study in *International Journal of Cardiology*, O Sullivan *et al*. did microRNA profiling from plasma samples from a cohort of control subjects, patients with stable coronary artery disease (CAD) and patients presenting with ST-elevation myocardial infarction (STEMI). The authors used a set of selected 22 miRs based on miRs with known role in cardiovascular biology, and identified four miRs (miR15a-5p, miR16-5p, miR93-5p and miR146a-5p) that were differentially expressed between controls subjects versus patients with stable CAD. Out of these miRs, miR 146a-5p was significantly decreased in stable CAD *vs*. control, while the rest of the miRs were found to be elevated in stable CAD compared to controls. The authors also found one miR (miR499a-5p) that was significantly elevated in patients with STEMI compared to control subjects. To adjust for traditional risk factors, the authors used a stepwise logistic regression model using all Framingham Heart Study (FHS) risk factors, and miR-93-5p remained significantly different between controls *vs*. stable CAD groups. Similarly, using a stepwise logistic regression incorporation using all FHS risk factors, the authors found miR-499a-5p was significantly elevated in patients with STEMI compared to controls. Further analysis using ROC curves showed that all four miRs that differed between control *vs*. stable CAD groups were significant predictors of stable CAD (AUCs of 0.67, 0.65, 0.68), while the miR-93-5p was found to be a better predictor based on the AUC curve of 0.75. In addition, addition of miR-93-5p to the FHS risk factors enhanced the discriminatory ability of FHS risk factors model to detect stable CAD. Similarly, the discriminatory ability of the FHS risk factors to detect STEMI was significantly enhanced with the addition of miR-499a -5p to the model.

This study by O Sullivan *et al*. provides an exciting step towards identification of potential circulating biomarkers for CAD. However, several notes of caution need to be considered. In the comparisons made by O Sullivan *et al*., the miRs were quantitated using "normalized expression" levels, adjusted to the average Cp of all expressed miRs, and therefore do not reflect absolute copy numbers. Given the prediction models were done using relative levels of miRs in plasma, it is possible that the prediction models may differ

based on the normalization method used.

An interesting finding was the value of miR93-5p expression to the traditional FHS risk factors to improve ability to detect stable CAD. This can be clinically useful and warrants further evaluation. It is interesting that the finding did not hold in control *vs*. STEMI subjects, and the significance of this remains unknown. STEMI is a diagnosis made based on clinical presentation and EKG-based criteria, and given the time constraints of definitive treatment for STEMI (8,9); the utility of a blood test is very limited, except for unusual cases where diagnosis can be confounded. Even in these situations, given the very high sensitivity and specificity of the currently available biomarkers for myocardial injury such as cardiac troponins (10), the potential clinical utility of miR-499a-5p is questionable. An ideal biomarker should provide diagnostic and prognostic information that is specific and incremental to existing clinical and demographic data, and in the context of STEMI, the findings from miR-499a-5p falls short of existing cardiac biomarkers. miR-499 is encoded by an intronic region of the myosin heavy chain gene (11). Therefore, circulating levels of miR-499 likely reflect the pathogenic process of myocardial damage. Given that the comparisons in this study were specifically made between controls and patients with STEMI, it is unclear if miR-499 is specific to STEMI, or a reflection of myocyte injury in response to any form of acute myocardial infarctions. Given similar findings of miR-499 from other studies in patients with acute myocardial infarction (12-15), miR-499 may have some clinical utility in detecting any form of myocardial infarctions at a time frame before cardiac troponins are detectable as shown by Wang *et al*. (15), or if a troponin negative, but miR-499 positive group is established to have true myocardial damage.

In selecting a panel of limited miRs with known role in cardiovascular disease, O Sullivan *et al*. used a candidate biomarker approach in this study. This approach allows for stronger statistical analysis of the selected miRs, but this limits the identification of potential unknown or novel biomarkers. In addition, the possibility that combined changes in a panel of miRs may have a better predictive ability for diagnosis of CAD over a single miR was not explored in the current study.

Finally, miRs in circulation can exist as free micro-RNAs, in exosomes or micro-particles, in protein-bound complexes, or in lipid complexes (16). In this study by O Sullivan *et al*., the authors examined the total circulating miRs, but it is prudent to consider that micro-RNAs from each of these circulating fractions may give different information compared to miRs from total circulating fraction. miRs from these fractions may provide alternate approaches to identify circulating miRs as biomarkers, and give crucial information regarding pathophysiology of a disease process.

A biomarker is a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions (17). Circulating miRs hold promise as potential biomarkers that can be indicative of these processes, but as knowledge of different circulating forms of miRs are still evolving, the identification of an ideal miR as a biomarker of CAD remains a work in progress.

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Footnote

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Sarco"MiR" friend or foe: a perspective on the mechanisms of doxorubicin-induced cardiomyopathy

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Provenance: This is a Guest Perspective commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Abstract: Anthracyclines are a class of chemotherapeutics used to treat a variety of human cancers including both solid tumors such as breast, ovarian, and lung, as well as malignancies of the blood including leukemia and lymphoma. Despite being extremely effective anti-cancer agents, the application of these drugs is offset by side effects, most notably cardiotoxicity. Many patients treated with doxorubicin (DOX), one of the most common anthracyclines used in oncology, will develop radiographic signs and/or symptoms of cardiomyopathy. Since more and more patients treated with these drugs are surviving their malignancies and manifesting with heart disease, there is particular interest in understanding the mechanisms of anthracycline-induced injury and developing ways to prevent and treat its most feared complication, heart failure. MicroRNAs (miRNAs) are small noncoding RNAs that regulate the expression of mRNAs. Since miRNAs can regulate many mRNAs in a single network they tend to play a crucial role in the pathogenesis of several diseases, including heart failure. Here we present a perspective on a recent work by Roca-Alonso and colleagues who demonstrate a cardioprotective function of the miR-30 family members following DOX-induced cardiac injury. They provide evidence for direct targeting of these miRNAs on key elements of the β-adrenergic pathway and further show that this interaction regulates cardiac function and apoptosis. These experiments deliver fresh insights into the biology of toxin-induced cardiomyopathy and suggest the potential for novel therapeutic targets.

Keywords: Doxorubicin (DOX); heart failure; microRNA (miRNAs); beta-adrenergic pathway

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Introduction to the biology of anthracycline toxicity in the heart

Anthracyclines, including doxorubicin (DOX), are among the most effective chemotherapeutic agents used to treat many human cancers. Unfortunately, their use is limited by adverse side effects, most notably dose-dependent cardiotoxicity. These events can manifest acutely following administration of the drug, or more commonly, months to years following cumulative exposure (1). In adults, early and late toxicity typically presents with dilated cardiomyopathy (2). Up to 5–10% of patients exposed to these drugs can have symptomatic or radiographic evidence of heart failure (3). Even more, some studies have demonstrated an increased risk of cardiotoxicity in those patients receiving low doses of DOX (<300 mg/m^2) which were once thought to be considered safe (4). Children, on the other hand, tend to present with dilated cardiomyopathy that progresses to a restrictive pattern (2). Childhood cancer survivors exposed to anthracyclines can have up to a 2–5 fold increased risk of heart failure compared to those patients not exposed to these drugs (5). Given the increasing number of cancer survivors amongst adults and children, anthracycline-induced cardiotoxicity is becoming a prevalent disease despite efforts dedicated to surveillance and prophylactic

management.

DOX is thought to deliver its anti-tumor effects primarily through inhibition of the alpha isoform of topoisomerase II (Top2α). The mechanisms of toxicity in the heart seem to be much more complex especially since cardiomyocytes lack expression of Top2α. These cells do, however, express the beta isoform (Top2β), and cardiomyocyte specific deletion of the gene encoding this protein confers protection against DOX-induced toxicity through inhibition of apoptosis and DNA-damage (6). Another mechanism of DOX-induced cardiotoxicity involves the generation of reactive oxygen species (ROS). This was originally a very attractive mechanism to explain cardiomyocyte injury as these cells have high metabolic rates, are chock full of mitochondria, and express low levels of anti-oxidant enzymes (7). Despite experimental evidence to support this theory, clinical efforts to scavenge free radicals have not been promising (8). Newer mechanisms have focused on the role of DOX in the inhibition of pro-survival pathways such as NRG-1/ErbB (9,10), and the stimulation of inflammation through Toll-like receptors (TLRs) (11).

MicroRNAs (miRNAs) are small noncoding RNA molecules that bind to the 3' un-translated region (UTR) of mRNAs and regulate their expression (12). Recently, miRNAs have been shown to play a critical role in many elements of cardiovascular disease including ischemia (13-15) and heart failure (16-18). Naturally, this led many groups to investigate the potential role of miRNAs in the development of DOX-induced cardiomyopathy (10,19-23). Roca-Alonso and colleagues continued this mission with a comparison of the global changes in miRNA expression in adult rat ventricular cardiomyocytes (ARVCMs) through two models. The first was an acute *in vitro* model of cultured ARVCMs harvested 6 hours after a single dose of DOX. The second was a chronic *in vivo* model where rats were exposed to repeated doses of DOX (cumulative dose of 15 mg/kg) over a two week period followed by harvesting ARVCMs 3 weeks later. A reference model of cardiomyopathy generated from rats with proximal left anterior descending (LAD) artery ligation was also included in the comparison. Three members of the miR-30 family (miR-30a, miR-30d, and miR-30e) were down-regulated in at least two of three models (21). Down-regulation of miR-30b has also been documented in H9C2 rat cardiomyocytes following hypoxia/re-oxygenation (24). Interestingly, other groups that generated cardiac miRNA profiles in animal models exposed to DOX had unique signatures that did not uncover miR-30 family members as being significantly dis-regulated (25,26). Nonetheless, there is quite a bit of variability in the timing of DOX exposure, the genetic background, and the technology used to generate these profiles.

The contribution of miR-30 and the β-adrenergic pathway towards the pathogenesis of heart failure

In cancer biology, miR-30 has been implicated as both an oncogene and a tumor suppressor. Its specific role tends to be cancer type specific (27-29). In the heart, overexpression of miR-30 directly regulated key pro-fibrotic proteins and thus may be associated with preventing the fibrosis characteristic of failing hearts (30). Another group demonstrated that up-regulation of miR-30 in cardiomyocytes blocked the up-regulation of angiotensin II-induced hypertrophy related genes and showed that increasing circulating levels of miR-30 may be used to diagnose myocardial hypertrophy (31).

Roca-Alonso and colleagues attempt to further promote miR-30 as a cardioprotective miRNA through a unique mechanism. Among the list of computationally derived predictive targets of miR-30, this group focused on three proteins in the β-adrenergic pathway (β_1AR, β_2AR, and $G_{i\alpha-2}$) (21). Modulation of contractile function in the heart via the β-adrenergic pathway involves the interaction of β_1AR and β_2AR with stimulatory guanylyl nucleotide binding proteins, Gs. This leads to the activation of adenylyl cyclase, an increase in cyclic AMP (cAMP), activation of protein kinase A (PKA), and the phosphorylation of direct components of the contractile apparatus and elements of the excitation contraction coupling system. β_2AR is also able to interact with inhibitory G proteins, G_i, which block adenylyl cyclase function as well as having the potential to activate the pro-survival phosphoinositide 3 kinase (PI3K)/Akt pathway (32-34). The role of the β-adrenergic pathway in the pathogenesis of heart failure has been well studied (32,34,35) and thus the choice to focus on β_1AR, β_2AR, and $G_{i\alpha-2}$ for further analysis was well conceived.

Chronic adrenergic stimulation in the heart has been shown to elicit cardiotoxicity (36). This effect is thought to be mediated primarily through aberrant activity of β_1AR resulting in calcium overload and cell death (37,38). In failing hearts, there is a down-regulation of β_1AR along with desensitization, whereas the density of β_2AR remains relatively unchanged (39). Transgenic mice overexpressing β_1AR specifically in cardiomyocytes developed fibrosis,

hypertrophy, and reduced fractional shortening at least in part due to increased apoptosis (37). Furthermore, administration of beta-blockers is the hallmark of heart failure treatment and prevention including heart failure from DOX-induced cardiomyopathy (8,40,41). On the other hand, β_2AR is thought to confer cardioprotection (38,42). This pro-survival phenotype is thought to be at least in part associated with its interaction with G_i proteins (43). In human end stage heart failure patients, G_i proteins, particularly the α-2 subunit ($G_{1\alpha\text{-}2}$), are up-regulated (44). Down-regulation of $G_{1\alpha\text{-}2}$ is associated with apoptosis and worsening heart failure (45-47). Activation of $G_{1\alpha\text{-}2}$ is slightly more controversial as one group demonstrated that constitutive activation of $G_{1\alpha\text{-}2}$ in a dilated cardiomyopathy and an isoproterenol-induced heart failure mouse model led to worsening hypertrophy and fibrosis, respectively. The authors postulate that the role of $G_{1\alpha\text{-}2}$ in fibroblasts as opposed to cardiomyocytes may be the driving force behind these phenotypes (43). To complicate the story even more, recent models have demonstrated reverse phenotypes for the role of β_1AR and β_2AR in heart failure whereby β_1AR is cardioprotective and β_2AR promotes cardiotoxicity (48). As a result, in addition to cell and disease specific contexts, the balance between β_1AR and β_2AR expression may contribute to the relative role of these receptors in the pathogenesis of heart failure.

The cardioprotective potential of miR-30

Since miRNAs typically have multiple mRNA targets, these molecules are poised to regulate the delicate balance of multiple effectors in a single influential pathway such as β-adrenergic signaling. Roca-Alonso and colleagues utilized luciferase assays in H9C2 cells to demonstrate direct binding of a miR-30e mimic to the wild-type 3' UTR of four predicted targets (β_1AR, β_2AR, $G_{1\alpha\text{-}2}$, and the pro-apoptotic protein E1B-interacting protein 3-like or BNIP3L) but not to mutant constructs. In addition, these miR-30e mimics were sufficient to attenuate the up-regulation of these targets upon administration of DOX (21). Appropriate controls with random sequence molecules were used throughout their experiments. These results support the author's claim that DOX-induced repression of miR-30 is at least partially responsible for increased expression of β_1AR, β_2AR, $G_{1\alpha\text{-}2}$, and BNIP3L.

In order to demonstrate that miR-30 is able to augment the downstream effects of the β-adrenergic pathway, Roca-Alonso and colleagues showed that DOX treatment or administration of a miR-30 sponge vector led to an increase in cAMP, although the magnitude of up-regulation was more prominent in DOX treated cells. This could be due to miR-30 independent mechanisms of cAMP signaling or incomplete miR-30 targeting, which is consistent with the fact that the authors demonstrate close to a 50% knockdown of miR-30e with sponge vectors. Overexpression of miR-30, on the other hand, resulted in decreased cAMP levels (21).

The phenotypic response to miR-30 was assayed through its effects on contractile function and DOX toxicity. Cells overexpressing miR-30 had an attenuation of contractile amplitude in response to increasing concentrations of isoproterenol compared to control cells. With regards to DOX toxicity, over-expression of miR-30 attenuated the increase in caspase activity triggered by DOX, although this attenuation was also incomplete. In addition, down-regulation of miR-30 led to an increased level of caspase activity compared to controls. Furthermore, in the presence of DOX, the intensity of ROS in cardiac cells was decreased with miR-30 overexpression and increased with miR-30 inhibition compared to cells exposed to DOX alone (21). While these findings are intriguing, they do not provide a direct link between miR-30 activity and damage through the β-adrenergic pathway. Future studies manipulating targets of miR-30 will be necessary to draw such conclusions.

Finally, Roca-Alonso and colleagues also uncovered the presence of GATA-6 binding to miR-30 cluster promoters in publically available data from chromatin immunoprecipitation sequencing experiments. They hypothesized that GATA-6 binding to these regions following DOX may mediate down-regulation of miR-30 (21). GATA-6 has already been implicated in cardiac pathology where it seems to be required to mount the cardiac hypertrophic response and prevent heart failure in animal models (49). To substantiate their hypothesis, these authors demonstrated that *GATA-6* is up-regulated acutely following exposure of DOX to cardiac cells in culture and siRNA constructs against *GATA-6* resulted in increased expression of miR-30 family members and decreased expression of miR-30 targets. Even more, in the setting of DOX treatment, knockdown of *GATA-6* resulted in decreased signaling through the apoptotic pathways (21). Nevertheless, experiments demonstrating the attenuation of miR-30 down-regulation after DOX in the absence of GATA-6 are lacking. As a result, other transcription factors could be more important than GATA-6 on the effect of DOX on miR-30. These experiments also beg the question of how GATA-6 is activated following DOX treatment and whether additional targets of GATA-6 could contribute to

the damage response. Moreover, if GATA-6 does indeed prove cardioprotective against failing hearts as demonstrated by others, its negative regulation of miR-30 would appear to promote cardiotoxicity. Further studies are needed to untangle the intricacies of these networks.

Roca-Alonso and colleagues provide an interesting model whereby down-regulation of β_1AR, β_2AR, and $G_{1\alpha\text{-}2}$ leads to a cardioprotective phenotype. While this certainly aligns with studies that demonstrate cardiotoxicity of excessive β_1AR, it appears to contradict the cardioprotective role of β_2AR and $G_{1\alpha\text{-}2}$. However, the authors offer an explanation of this apparent contradiction by arguing that the fine tuning of expression characteristic of miRNAs can regulate the feedback loop involving β_1AR, β_2AR, and $G_{1\alpha\text{-}2}$ in such a way to prevent the cardiotoxicity of β_1AR signaling, while still maintaining some of the cardioprotective benefits of β_2AR and $G_{1\alpha\text{-}2}$. In a similar fashion, the beta-blocker Carvedilol has been shown in patients and animal models to be protective against DOX-induced cardiomyopathy despite being characterized as nonselective towards β_1AR and β_2AR (40,41). Most of the experiments for these conclusions were based on *in vitro* cell cultures. Future studies *in vivo* are necessary to determine the long-term phenotypic consequences of miR-30 knockdown and overexpression on DOX-induced cardiomyopathy in order to fully test the therapeutic potential of these miRNAs. Such models can also be used to determine potential additive or synergistic benefit of miR-30 overexpression with beta-blockade.

Conclusions

In a recent report by Roca-Alonso and colleagues, a novel mechanism for DOX-induced injury involving the regulation of the β-adrenergic pathway through miRNAs is uncovered. These findings provide new insight into the complex pathways that govern the damage response and the delicate balance between the expression of key elements in a single pathway. Even more, these studies offer exciting potential for future therapeutic targets of chemotherapy-induced cardiomyopathy.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Molecular evidence that exercise training has beneficial effects on cardiac performance

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Numerous epidemiological and observational studies demonstrate that there is an inverse relationship between physical activity and risk of cardiovascular disease (1). Recently a few randomized controlled trials revealed that exercise training is not only effective as primary prevention, but also in the secondary prevention and thus can be viewed as a "medication" that should be taken on a regular basis by a patient with cardiovascular disease. Exercise in Left Ventricular Dysfunction (ELVD) trial in a small group of 77 patients with <40% ejection fraction after a first Q-wave myocardial infarction showed that a 6-month exercise training program prevented deleterious LV remodeling (2). The Heart Failure: A Controlled Trial Investigating Outcomes of Exercise Training (HF-ACTION) trial, enrolling 2,331 outpatients with stable systolic heart failure demonstrated that exercise training was associated with an 11% reduction in combined all-cause death or hospitalization (P=0.03) (3). A meta-analysis of exercise training trials in patients with chronic heart failure, majority of whom had a history of myocardial infarction, revealed that exercise training significantly reduced deaths and hospital admissions (4). Thus exercise training is recommended as part of a comprehensive approach to the patient with stable chronic heart failure (1).

Presumable mechanisms of this beneficial effect of exercise in the post-myocardial infarction and chronic heart failure setting include: improvement of VO2max, reduction of neurohormonal imbalance, antiarrhythmic effects, resolution of ventilatory abnormalities, improvement of endothelial function, improved both systolic and diastolic myocardial performance through improvement of cardiomyocyte contraction-relaxation cycle.

Cardiomyocyte contractile function has been shown to be impaired in post-MI heart failure. Decreased amplitude of myocyte contraction as well as slower kinetics of contraction-relaxation cycle has been demonstrated in many experimental models of post-MI heart failure and in humans (5,6). Cardiomyocyte contractile function is strictly controlled by beat-to-beat transient increase of intracellular Ca^{2+} concentration (i.e., calcium transient). After electrical activation, rising of the membrane potential opens the voltage-gated sarcolemmal L-type Ca^{2+} channels. This results in influx of small amount of Ca^{2+} to the myocyte, which activates the calcium-dependent sarcoplasmic reticulum (SR) Ca^{2+} release channels [ryanodine receptors (RyRs)]. This process is commonly called calcium-induced calcium release. Rapid release of considerable amount of SR Ca^{2+} results in increase of intracellular Ca^{2+} concentration and promotes Ca^{2+} binding to troponin C, a contractile apparatus regulatory protein. The change of tropnin C conformation upon Ca^{2+} binding enables actin-myosin interaction and thus myocyte contraction. Relaxation is initiated by termination of the Ca^{2+} release from the SR and by rapid Ca^{2+} removal from the cytosol. Two main

transporting proteins are involved in this process: SR Ca^{2+}-ATP-ase (SERCA) which uses ATP to pump calcium back into the SR and the Na^{+}/Ca^{2+} exchanger (NCX) which transports 1 Ca^{2+} ion out of the cell and 3 Na^{+} ions into the cell. SERCA accounts for approximately 80% of removal of systolic calcium in humans and even more (about 90%) in rodents Thus, the transporting function of SERCA is the main determinant of the rate of cardiomyocyte relaxation. Additionally the SERCA transporting ability determinates the SR Ca^{2+} content and thus amplitude of Ca^{2+} transient and amplitude of myocyte contraction. The transporting function of SERCA depends on its expression, intrinsic activity of enzyme (ability to utilize ATP) and the phosphorylation level of phospholamban, endogenous SERCA inhibitor. Phospholamban is phosphorylated at Ser-16 and Thr-17 by adrenergic stimulation dependent kinase (PKA) and calmodulin and Ca^{2+} dependent kinase (CAMKII), respectively and increase of phosphorylation level relives SERCA inhibition (7).

In post-MI heart failure detrimental changes in Ca^{2+} handling have been described. Decreased SERCA transporting function has been demonstrated consistently, due to both decreases of SERCA expression as well as decreased transporting ability, mainly due to reduced level of phospholamban phosphorylation. Moreover, in many animal models and in humans increased NCX expression or/and function has also been described. These changes are additionally accompanied by an increased Ca^{2+} sensitivity of RyRs due to their hyperphosphorylation which results in Ca^{2+}-leak from the SR independently from Ca^{2+} influx through the L-type Ca^{2+} channels (diastolic Ca^{2+}-leak) (8,9).

Decreased SERCA expression accompanied by increased NCX function results in increased proportion of intracellular Ca^{2+} removed from the cytoplasm by NCX as compared with SERCA. This together with increased Ca^{2+}-leak results in decreased Ca^{2+} SR content, amplitude of Ca^{2+} transient and myocyte shortening as well as the decreased rate of Ca^{2+} transient decay and slower relaxation. Moreover, increased NCX contribution to the relaxation increased inward current (1 Ca^{2+} ion is exchanged with 3 Na^{+} ions) and may promote afterdepolarization, premature beets and increased susceptibility to ventricular arrhythmias. Indeed the post-MI animals as well as patients with ischemic heart failure die from progressive pump failure or sudden arrhythmic events (10).

Many elegant papers have shown that regular, intensive aerobic exercise training influences Ca^{2+} handling and thus myocyte contraction and relaxation process in cardiomyocytes from both healthy and post-MI hearts (11).

In healthy animals exercise training resulted in approximately 30% increase of transporting activity of SERCA measured in the intact SR membranes or permeabilized cardiomyocytes (12). It was due to increased SERCA expression at mRNA and protein level as well as increased phospholamban phosphorylation. Additionally in some studies increased level of NCX and increased sensitivity of contractile apparatus were observed. Consequently, amplitude of the cell shortening and the rate of relaxation were increased (13).

In post-MI heart failure, exercise training seems to be especially beneficial. The restoration of the normal amplitude and rates of contraction and relaxation has been observed. It was associated with normalization of the expression of SERCA and NCX proteins. It supports the cardiomyocyte function and decrease propensity to Ca^{2+}-dependent ventricular arrhythmias (14). There is growing body of evidence indicating that exercise training is able to restore of the proper expression of the protein involved in Ca^{2+} handling in filing hearts the mechanisms of this restoration is still poorly understood.

The discovery of microRNAs (miRNAs), abundant single-stranded small (roughly 22 nucleotide long) nonprotein-coding RNAs, has made important contribution to the better understanding of mechanisms that regulate of gene expression. MicroRNAs have been shown to be involved in most biological processes, both physiological and pathophysiological, including cardiovascular diseases. MicroRNAs are transcribed as individual or in clusters, often as part of longer transcripts, and are expressed in a tissue and cell-specific manner. The miRNA system is generally regarded as a negative regulator of specific mRNA targets. They can inhibit translation and/or promote mRNA degradation by sequence-specific base pairing (15).

Many well documented studies revealed that microRNAs were frequently downregulated in various types of cardiac diseases, including pathogenesis of MI (16). Because miRNAs are important in many cardiac pathologies, they may play a functional role in exercise-induced cardiac phenotypes.

Melo *et al.* in their work titled "*Exercise training restores the cardiac microRNA-1 and -214 levels regulating* Ca^{2+} *handling after myocardial infarction*", published in *BMC Cardiovascular Disorders* (17) demonstrated that myocardial infarction in the rat resulted in reduced expression of SERCA and increased expression of NCX. Expression of microRNA-214 that targets SERCA, was increased, white

that of microRNA-1 that targets NCX, was reduced. Ten weeks of exercise training resulted in restoration of both microRNA levels and prevents changes of expression of both calcium transporters induced by myocardial infarction. These results suggest that changes in microRNA are responsible for restoration of SERCA and NCX expression, through this conclusion is only based on the above mentioned correlations.

This article provides new data on possible mechanisms behind effects of exercise training on cardiac performance in infracted heart. microRNA-1 is cardiac specific miRNA and plays a role in heart hypertrophy, myocardial infarction, and arrhythmias, by promoting apoptosis. Recently, studies have revealed that miR-1 was frequently downregulated in various types of cardiac disease but when overexpressed, played a protective role against cardiac hypertrophy or heart failure by regulating several hypertrophy-associated genes, including transcription factors, receptor ligands, apoptosis regulators and ion channels (18). Overexpression of microRNA-1 and other miRs is implicated in regulation of G-PCR and calcium handling (19). Second miRNA investigated by authors, microRNA-214, improved LV remodeling and decreased apoptosis of myocardial cell and had a protective effect on heart function (20).

In summary, the study by Melo *et al*. highlights new potential mechanisms of beneficial effects of exercise on the post-MI heart, providing new areas for future research.

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Footnote

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How to be young at heart? miR-22 as a potential therapeutic target to boost autophagy and protect the old myocardium

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Cardiovascular diseases remain the most common cause of death in Western countries and their prevalence in the general population increases progressively with age, reaching more than 60% in subjects older than 60 years (1). This is at least in part due to a progressive impairment of the cellular processes regulating cardiac and vascular homeostasis, finally leading to the development of cardiovascular abnormalities (2). In addition, the molecular mechanisms that protect the heart against stress are downregulated by aging, making the myocardium more susceptible to injury (2). It is therefore important to find new therapeutic targets to reduce cardiovascular aging and protect the aged myocardium from stress.

Autophagy is a mechanism of intracellular degradation through lysosomes characterized by the presence of double membrane vesicles called autophagosomes (2,3). Increasing lines of evidence suggest that autophagy plays an important role in maintaining cardiac homeostasis. Autophagy is downregulated in the heart during aging, and downregulation of autophagy plays a causative role in the progression of aging in the heart (2,4). Furthermore, we and others have shown that downregulation of autophagy during pressure overload (5,6), post-myocardial infarction cardiac remodeling (7) and metabolic syndrome (8,9) contributes to the progression of cardiac dysfunction and ischemic injury. On the other hand, excessive activation of autophagy during the acute phase of myocardial infarction or ischemia/reperfusion may be detrimental (10). Thus, identifying molecular interventions to control the activity of autophagy is important and may lead to the development of a novel treatment for heart disease, especially in elderly patients.

Using high-throughput screening based on the fluorescence-activated cell sorting-based detection of GFP-LC3 protein and library screening, Gupta *et al*. identified miR-22 as a strong inhibitor of autophagy in cardiomyocytes (11). The authors showed that miR-22 inhibits the reduction in GFP-LC3 signal in response to starvation, an established stimulus for autophagy. This screening method is based on the fact that the GFP-LC3 signal is attenuated when GFP-LC3 autophagosomes move from the cytosol to lysosomes (12). Thus, it is a convenient and reliable method to evaluate the level of autophagic flux from autophagosome formation to autolysosome formation. A cautionary note, however, is that the results of this method are easily affected by conditions that alter the intensity of the GFP signal through autophagy-independent mechanisms. Thus, the authors could have conducted more extensive validation regarding how miR-22 affects autophagic flux in cardiomyocytes throughout the study. Nevertheless, this is a fascinating way to identify effective modifiers of autophagy in cardiomyocytes and, by extending this approach to small molecules, it may be possible to identify novel interventions for heart failure patients.

Gupta *et al*. demonstrated that miR-22 expression progressively increases in the mouse heart during aging (11). miR-22 inhibition by locked nucleic acid-based anti-miR-22 (LNA-anti-miR-22) significantly attenuated cardiac remodeling in old mice subjected to permanent coronary artery ligation, reducing left ventricular dilation and improving systolic function. In contrast, the beneficial effects of miR-22 inhibition were not obvious in young mice with chronic myocardial infarction. Thus, miR-22 upregulation in the aged heart appears to impair adaptation to chronic myocardial infarction. miR-22 inhibition limits senescence-induced cardiomyocyte hypertrophy in vitro. Therefore, the cardioprotective effects of pharmacological miR-22 inhibition in the mouse heart may be dependent on its protective actions in cardiomyocytes. However, since miR-22 also induces senescence in cardiac fibroblasts, increasing their migratory capacity (13), it is possible that miR-22 inhibition may also elicit beneficial functions through the other cell types in the heart.

miR-22 appeared to be responsible for the reduction of autophagy in the old myocardium. The increase in cardiac miR-22 levels during aging was paralleled by a progressive decline in myocardial autophagy. Importantly, miR-22 inhibition appears to rescue autophagy in aging hearts (11). miR-22 may have autophagy-independent actions, and thus, the causative involvement of autophagy in mediating the salutary actions of miR-22 inhibition remains to be demonstrated. Nevertheless, the fact that miR-22 inhibition can stimulate autophagy in the aging heart is attractive. Restoring the level of autophagy has been shown to be salutary in some cardiovascular conditions. For example, genetic inhibition of the serine-threonine kinase MST1, a stress activated kinase known to inhibit autophagy, reduces remodeling caused by chronic ischemia through the activation of autophagy (7). In addition, administration of spermidine, a natural polyamine compound, extended life span in mice and reduced cardiac hypertrophy and diastolic dysfunction induced by aging. These effects were found to be dependent on the capacity of spermidine to activate autophagy through the inhibition of the histone acetyltransferase p300. In fact, the cardioprotective effects of spermidine were lost in mice with genetic disruption of autophagy (4).

Other miRs also regulate autophagy in the heart. Thum's group elegantly showed that miRNA-212/132 inhibits autophagy in cardiomyocytes by targeting FoxO3a. Pharmacological inhibition of miR-132 reduced cardiac hypertrophy and heart failure induced by pressure overload (14). In addition, exosome-mediated miR-145 administration was found to reduce ischemic injury by activating autophagy (15). Thus, targeting these miRNAs could represent a new avenue of exploration in the development of novel interventions to treat heart failure in elderly patients.

miR-22a plays a dual role in the heart, performing both physiological and maladaptive functions. Mice with either systemic or cardiac-specific miR-22 gene deletion do not develop compensatory hypertrophy in response to isoproterenol treatment and display increased cardiac dilation and dysfunction with respect to control mice (16). Similarly, systemic miR-22 knockout mice were found to develop cardiac dilation and dysfunction in response to pressure overload (17). These effects were associated with a downregulation of Serca2a expression and with sarcomere disarray. Interestingly, however, miR-22 gain of function also appears to be maladaptive. Mice with cardiac-specific miR-22 overexpression develop cardiac hypertrophy and dysfunction, and these deleterious effects are associated with impaired calcium handling and reduced expression of PPARα, SIRT1 and PGC-1α (18). Thus, it is likely that proper cardiac function requires that miR-22 expression levels remain within a relatively narrow physiological range.

The molecular mechanism by which miR-22 inhibits autophagy has not yet been fully elucidated. miR-22 was found to inhibit autophagy in cardiomyocytes by reducing the expression of PPARα (11). However, other mechanisms may also be involved in the inhibitory effects of miR-22 on autophagy. It was previously demonstrated that miR-22 reduces the expression of PTEN, a negative regulator of the AKT pathway, in cardiomyocytes (19). This suggests that miR-22 may also suppress autophagy through activation of AKT, which may, in turn, activate the kinase mTOR, a negative regulator of the autophagic process (20). Since miR-22 affects cardiac function through multiple mechanisms, it is also possible that the inhibition of autophagy is indirectly mediated through other functional targets of miR-22, such as the mitochondrial dysfunction and metabolic remodeling observed in hearts following myocardial infarction. Future studies are warranted to test this possibility.

Aside from autophagy, other mechanisms may also underlie the beneficial cardiac effects of pharmacological miR-22 inhibition (*Figure 1*). Inhibition of the AKT/mTOR pathway may be one of these mechanisms, since

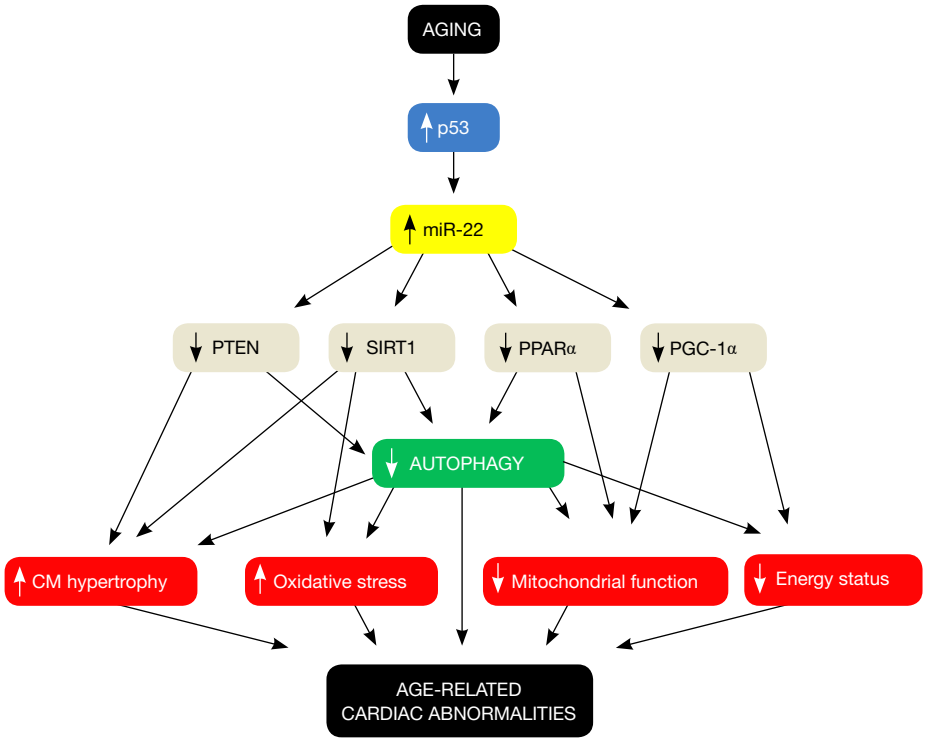

Figure 1 miR-22 promotes age-related cardiac abnormalities. Schematic representation of the molecular mechanisms through which miR-22 promotes age-related cardiac abnormalities. Autophagy inhibition appears to play a major role in the development of these abnormalities. CM, cardiomyocyte; PTEN, phosphatase and tensin homolog; PPARα, peroxisome proliferator-activated receptor α; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-related protein 1α.

there is a large body of evidence demonstrating that chronic and deregulated activation of mTOR is detrimental during cardiac stress, whereas its pharmacological inhibition is protective (20). SIRT1 was also found to be a target of miR-22 (18), so that activation of SIRT1 may represent another mechanism through which miR-22 inhibition may reduce age-related cardiac diseases. We previously found that SIRT1 reduces cardiac aging and confers resistance to oxidative stress (21), and SIRT1 has also been shown to limit ischemic injury (22). Finally, miR-22 inhibition may upregulate PGC-1α, another miR-22 target. PGC-1α controls mitochondrial biogenesis and its upregulation may favor mitochondrial turnover and proper mitochondrial function, which are usually impaired in the old heart (2). In this regard, mice with PGC-1α gene deletion display an impaired energy state and develop contractile dysfunction during aging (23).

One of the most remarkable results of the study by Gupta *et al*. is the demonstration that miR-22 dysregulation is relevant to human disease. In fact, the authors found that high circulating levels of miR-22 are associated with a higher cardiovascular mortality in patients affected by heart failure (11). Future studies are warranted to investigate whether miR-22 can be used as a biomarker to monitor the efficacy of pharmacological therapy in heart failure patients. It will be important to evaluate whether the pharmacological inhibition of miR-22 ameliorates symptoms and outcomes in these subjects. Of note, atorvastatin was previously seen to reduce the expression of miR-22 in cardiomyocytes (24) and was also shown to ameliorate heart failure in patients (25).

In conclusion, Gupta *et al*. provided compelling results indicating that miR-22 upregulation in the heart is responsible for the reduction of autophagy and the increased susceptibility to stress during aging. miR-22 may be considered as a potential therapeutic target for the treatment of age-related diseases (*Figure 1*).

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Footnote

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Another promise against ischemia reperfusion injury: every success raises new questions

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Comment on: Bian C, Xu T, Zhu H, *et al.* Luteolin Inhibits Ischemia/Reperfusion-Induced Myocardial Injury in Rats via Downregulation of microRNA-208b-3p. PLoS One 2015;10:e0144877.

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The study by Bian *et al*. (1) raises many important deliberations. Briefly, they found that luteolin (LUT), a flavonoid found in many vegetables, fruits and seeds, inhibits ischemia/reperfusion induced myocardial injury (IRI) in rats.

The first question is: how relevant to the clinical situation are results on IRI alleviation in the experimental setting. The number of successful interventions in animals is legion. However, against these promising results, the very important position paper of the Working Group of Cellular Biology of the Heart of the European Society of Cardiology (2) should be remembered: the experts producing this paper concluded only 3 years ago that there is no effective proven therapy against IRI. It is widely recognized that it is not always possible to translate animal experiments into clinical therapy.

According to Bian *et al*. (1), LUT joins a long list of herbal medicines proposed to have wonder qualities, such as quercetin, curcumin, resveratrol, and many others. LUT is followed by an impressive list of references, supporting it as an antineoplastic and cardioprotective agent. In its latter role LUT has been given only in rodents, mostly on isolated cardiomyocytes (3,4) or hearts under Langendorff perfusion as in the present study (3,5) or both (4,6). Sun *et al*. (7) used the drug in diabetic rats undergoing coronary artery occlusion for 30 minutes followed by 3 hours of reperfusion. The drug was administered via tail vein injection, which makes it a feasible agent for employment in the clinical arena as an adjunct to primary percutaneous coronary intervention.

Many mechanisms have been proposed for the cardioprotective effects of LUT against IRI. Thus in previous studies, Qi *et al*. (8) from the same group found that LUT decreased both necrosis and apoptosis. As regards apoptosis, they found that LUT upregulated Bcl-2, decreased the ratio of Bax to Bcl-2 and inhibited the activation of caspase-3. These findings are important since IRI engenders both necrosis and apoptosis (9). Xu *et al*. (3) again from the same group in a review recapitulate the action of LUT on the following processes, involved in apoptosis: upregulation of phosphorylated Akt, suppression of NF-κF activation, increase of Bcl-2, inhibition of caspase-8 and -3.

Fang *et al*. (4) additionally reported that LUT increased phosphorylated SERCA-2 and phospholamban as opposed to control, through the p13K/Akt pathway. Also, Wu *et al*. (6) found that it activates pERK and inhibits the JNK pathway. Reduction of JNK, and p38 MAPK are also reported by Cheng *et al*. (10) in rat cortical necrosis.

Sun *et al*. (7) also showed that LUT inhibits LDH release (suggestive of necrosis inhibition) and in addition to its decrease of the Bax to Bcl-2 ratio, it upregulated the anti-apoptotic proteins FGFR2 and LIF and increased BAD phosphorylation. It also inhibited MPO expression and inflammatory cytokine production, including IL-6, IL-1a and TNFα. They also found that LUT decreased

infarct size as measured by Evans Blue/TTC staining, and myocardial apoptosis, as assessed by TUNEL, while improving cardiac function as assessed by echocardiography, and the incidence of arrhythmia.

In the index study, Bian *et al*. (1) studied Langendorff perfused rat hearts, subjected to ischemia for 30 min and reperfused for 120 min, in which cardiac function was studied with a pressure sensor Millar catheter. They found that LUT pretreatment in the perfusate improved cardiac function and also had the following effects on microRNAs (miRs): it downregulated the expression of 4 miRs, including miR 208b-3p and upregulated the expression of 26 miRs.

Additionally, they transfected the H9c2 cells with a specific miR-208b-3p mimic or a duplex RNA inhibitor to effectively overexpress or knock down miR 208b-3p.

Then after the cells where transfected with a siRNA to knock down Ets-1, they underwent an anoxia/reoxygenation protocol (A/R simulation of I/R).

They showed that in the cell culture LUT-pretreatment protected the cells against A/R injury, reversing the appearance of dead cells, while significantly downregulating miR 208b-3p. They verified that by administrating the miR 208b-3p mimic; this miR was very robustly overexpressed with increased cell apoptosis, while it was greatly underexpressed when incubated with its inhibitor, with resulting decreased apoptosis.

Also, with the most effective Ets-1-SiRNA sequence, Ets-1 was strongly underexpressed, with an increase of H9c2 cells in the early phase of apoptosis.

The administration of LUT was accompanied by a corresponding decrease of pro-(caspase-3 and Bax) and increase of anti-(Bcl-2) apoptotic agents. The miR 208b-3p mimic decreased Ets-1 protein levels. These levels were however increased with LUT addition and miR-208b-3p inhibition.

These results give some important messages:

First, the decrease of miR-208b-3p expression by LUT is potentially of significance. This miR has been found to be increased after an acute myocardial infarction (11). The novel finding that its inhibition decreases apoptosis can have important clinical consequences. Equally importantly, miR-208b-3p has been associated with post infarct myocardial remodeling (REM), being one of the main miRs associated with this unfavorable course (12).

Thus, an agent, such as LUT could potentially affect a diminution of myocardial death both acutely, signifying cardioprotection, and chronically, signifying cardiopreservation: since LUT is easy to administer orally, a chronic experiment to evaluate its action against REM is in order.

When a miR is found effective in the living organisms, theories about its potential clinical value immediately arise. However, up to now the effectiveness of antagomirs given systematically is less than satisfactory (13); direct local infusion is needed. The same holds true for miR-mimetics, which need to be attached to lipoparticles or to viral vectors (5). Nanoparticle therapy clinically still belongs to the future.

Thus, the use of a simple substance which can readily and robustly manipulate miR expression is very promising.

A second finding attributed to LUT should not be overlooked. Through its diverse reported actions it has been found to decrease the incidence of myocardial infarction in the Zutphen elderly study (14). If this-as logically expected-could also include re-infarctions, LUT could protect patient populations with coronary artery disease against REM by decreasing re-infarctions, such as Kjekshus (15) has advocated to happen with the use of statins.

The study of Bian *et al*. (1) brings into focus another problem. The authors state that LUT increases Ets1 protein levels. The same result was seen with transfection with a miR-208b-3p inhibitor. Thus they suggest that Ets1 is a target gene of miR-208b-3p. They pertinently state that there exist relatively few reports on the role of Ets-1 on cardiomyocyte apoptosis. However, Wang *et al*. (16) found that in hyperglycemia, HMGB1 induces apoptosis via an ERK/Ets-1 pathway; moreover, caspase is its direct target gene (17).

Ets-1 can induce inflammation and apoptosis in endothelial cells (18,19) but reduce apoptosis in vascular smooth muscle cells (20) which share many properties with cardiomyocytes. Thus, the role of Ets1 on cardiomyocytes needs further study.

Lastly, I must point out another source of perplexity: LUT is advanced as being both cardioprotective through its inhibition of apoptosis of cardiac cells and anti-neoplastic through its promotion of apoptosis and inhibition of angiogenesis (21).

Cai *et al*. (21) showed that in human pancreatic carcinoma cells it increased Bax and caspase-3 and decreased Bcl-2 expression. These are exactly the opposite to what Qi *et al*. (8) and Fang *et al*. (4) have found in the rat heart.

Is the Yin and Yang of Tao philosophy taken too far? Invariably, transcriptional pathways follow similar courses in biological processes, such as cardiac, neoplastic and diabetic

system perturbations. Could the very intense proliferation rate of neoplastic cells and the marked quiescence of cardiomyocytes explain this difference? It is interesting that many cancer cells have higher than normal Akt (22) and Bcl-2 levels (23). Obviously these perplexing biological oddities should be studied further. They also often manifest downexpression of the apoptotic phosphatase PTEN (22), an antagonist of pro-survival PI3K which is highly expressed in cardiomyocytes, and inactivation of caspases (24). These differences may play a role.

Here, the author must confess that he is skeptical towards oriental herbal medications, which are advanced as a panacea (an all-curing medicine in ancient and modern Greek). Still one should never dismiss ideas which are not to his preference. It should not be overlooked that very recently, Eggebeen *et al*. (24) in JACC Heart Failure reported that beetroot juice has beneficial effects in older patients with heart failure and preserved ejection fraction. This is American herbal medicine at its best.

Thus, I would conclude that the elegant study of Bian *et al*. (1), a group consistently studying LUT over the years, preempts a host of clinical and theoretical considerations and definitely warrants further study, both acutely and also due to its ease of use against chronic cardiac REM, a subject on which this author has been working for over 20 years (25).

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None.

Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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RNAs that make a heart beat

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Comment on: Liu X, Zhang Y, Du W, *et al*. MiR-223-3p as a Novel MicroRNA Regulator of Expression of Voltage-Gated K+ Channel Kv4.2 in Acute Myocardial Infarction. Cell Physiol Biochem 2016;39:102-14.

Abstract: An increase in stress-associated microRNAs has been observed in the heart after an induced myocardial infarction. Liu and colleagues now demonstrate that one of these stress-associated microRNAs, *miR-223-3p*, can regulate a component of the voltage-gated channel that mediates rapid outward efflux of potassium during an action potential. Aberrations in the potassium current have been associated with ventricular arrhythmia and heart disease. Strikingly, introducing a small RNA antagonist directed against *miR-223-3p* into rat hearts, while also inducing a myocardial infarction, resulted in a reduction in arrhythmias. We place these studies in the larger context of the field and discuss the potential of anti-*miR-223-3p* molecules as new therapeutics for myocardial infarction.

Keywords: Action potential; potassium channel; voltage-gated channel; microRNA; arrhythmia; myocardial infarction

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Action potentials drive heart beat

The beating of a heart is a symbol of life and vitality. The ability of the heart to pump blood throughout the body is essential for bringing oxygen and nutrients to peripheral tissues and removing carbon dioxide and other wastes. Heart beats power this flow of blood. Each time the heart beats, arteries expand and fill with blood; the pauses between the beats allow for constriction followed by a re-expansion with the next heart beat.

The ability of the heart to beat relies on the conversion of electrical to mechanical energy (1). The electrical basis for heart beats are action potentials that start in the specialized pacemaker cells of the heart, and are then transmitted to the atrial and ventricular heart muscle cells, cardiac myocytes, via the passage of ions between cells through gap junctions. Action potentials involve the flow of ions into and out of cardiac myocytes through voltage-gated channels. These channels open and close depending on the membrane potential, the difference in electrical potential on the inside of the cell compared with the outside (*Figure 1*). During an action potential, the opening and closing of one set of channels leads to a change in membrane potential, thereby allowing a different set of channels to open. These changes lead to entrance or exit of different ions, and subsequent changes in transmembrane potential (3-7).

During cardiac action potentials, there is a flux of sodium (Na^+) into the cell, along with an inward flux of calcium (Ca^+), that leads to the depolarization, followed by outward potassium (K^+) currents that repolarize the cell (1,8-11). An action potential is divided into several phases (1,9-12). In phase 4, the resting phase, the membrane potential of a ventricular cardiomyocyte is about –90 mV because, like most cells, ventricular cardiomyocytes have a more negative charge inside than outside of the cell. The membrane potential is established largely by potassium channels that allow for a loss of positively charged potassium ions from the inside of the cell resulting in a negative membrane

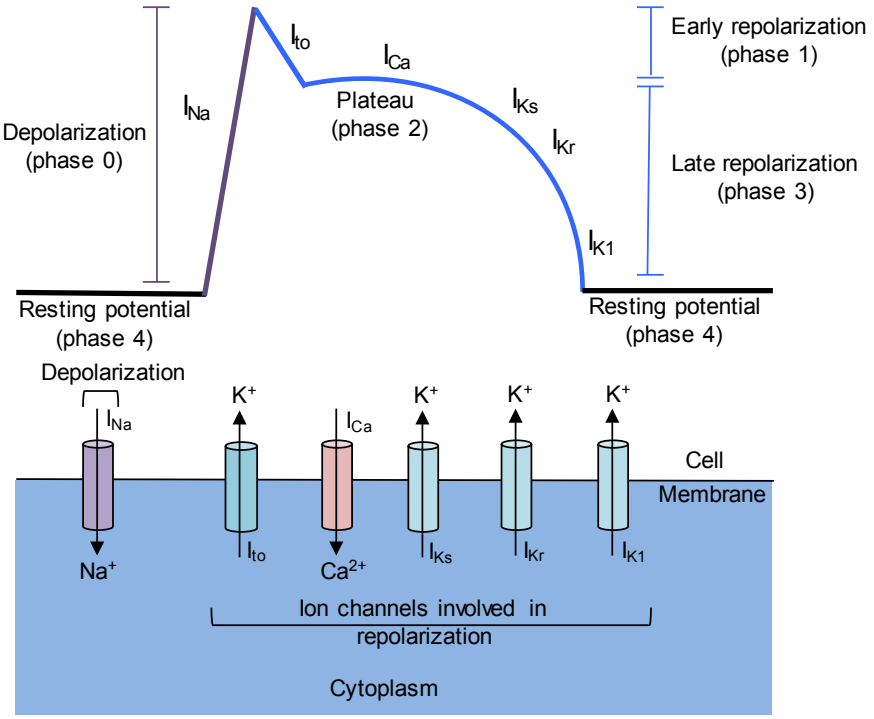

Figure 1 Example of a ventricular action potential. The membrane potential is plotted over time on the y axis. Different currents active at different phases of the action potential are labeled. Below, the different ion channels that open and close to produce an action potential are shown. Adapted with permission from Kim 2013 (2).

potential. In addition, sodium-potassium pumps actively transport three sodium ions out of the cell and two potassium ions into the cell, both against a concentration and electrical gradient, thus maintaining the concentrations of both ions and preserving the voltage polarization.

Phase 0 is the depolarization phase that initiates the action potential. An action potential can result from an electrical stimulation, or, in the case of the pacemaker cells of the heart, from spontaneous automaticity of the pacemaker cells due to a slowly depolarizing Na channel that generates a pacemaker current called the funny current (10). In ventricular myocytes, rapidly activating, phase 0, voltage-gated channels specific for sodium ions open. Sodium ions are more abundant outside than inside the cell, and opening these channels results in a rush of positively charged sodium ions into the cell (I_{Na}). The flooding of sodium ions changes the transmembrane potential from negative to positive, resulting in depolarization of the membrane to about +20 mV.

The subsequent phases involve a repolarization of the membrane as positive ions flow outward from the cell. In the next phase, phase 1, the sodium channels close and voltage-gated potassium (K_v) channels open, creating an outward potassium current I_{to}. The rapid efflux of positively charged potassium ions results in a sharp decrease in the membrane potential from ~+20 mV to ~+10 mV at the end of phase 1. Phase 1 of an action potential can influence the height and duration of the ensuing phases (12,13).

The membrane repolarization of phase 1 activates voltage-gated calcium currents (I_{Ca}). In phase 2, a plateau phase, there is a balance between a slow inward flux of calcium ions and a reduced outward flux of potassium ions through a different set of potassium channels, the slow delayed rectifier potassium channels (I_{Ks}). The membrane potential in phase 2 ends at ~–20 mV. The calcium ions that enter the cell in phase 2 act as triggers for contraction of the myofilaments of the heart muscle.

In phase 3, the calcium channels close. The rapid delayed rectifier potassium current (I_{Kr}) contributes to the rapid potassium efflux that creates phase 3 of repolarization. Finally, an inward delayed rectifier potassium current (I_{K1}) helps establish and maintain the cell transmembrane potential (phase 4). The I_{K1} current represents the current that must be overcome to establish the next action potential.

A cardiac action potential lasts ~200 msec. After an action potential, there is a refractory period of ~250 msec in which another action potential is unable or less likely to occur. After the refractory period, the cell returns to the resting phase and can respond to signals that evoke a subsequent action potential.

Voltage-gated potassium channels are tightly regulated to control myocyte function and arrhythmia

Voltage-gated channels allow fast and selective ion permeation that is regulated by opening and closing of a pore through a mechanism that senses transmembrane voltage (14). Voltage-gated channels are composed of alpha pore-forming subunits and accessory subunits (1,15). The pore-forming alpha subunits contain multiple transmembrane domains. The channels can be homotetrameric or heterotetrameric with four voltage-sensitive alpha subunits arranged surrounding a common center that, when open, serves as a pore for ions (15).

The K_v channel has six transmembrane domains, with both amino and carboxy termini localized within the cell (14). The two transmembrane domains at the carboxy terminus of the protein form the pore for potassium ion flux. The transmembrane domain adjacent to the pore pore-forming domains contains positively charged arginine and lysine residues and moves in response to membrane depolarization, thereby mediating the voltage-dependent opening of the channel (15).

Through extensive gene duplication events, the human genome encodes 40 voltage-gated potassium channels (12). The expression and regulation of these potassium channels, for instance, in different positions within the heart, can lead to changes in action potential amplitude, duration, waveforms, and rhythmicity (8,12). The transient voltage-gated potassium current (I_{to}) in phase one is sometimes divided into $I_{to,fast}$ and $I_{to,slow}$ based on the rate of recovery (20–100 ms for the fast current and seconds for the slow current) (11). The pore-forming subunits of $I_{to,fast}$ are $K_v4.2$ and $K_v4.3$, which are encoded by the *KCND2* and *KCND3* genes. These proteins can likely form pores as homomers or heteromers (16,17).

Heart beats that deviate from the normal rhythm are called arrhythmias (11). Heart failure, a common cause of death worldwide, is frequently associated with arrhythmias and electrical instability. Abnormalities in the repolarization phase of action potentials due to heart failure can contribute to arrhythmias (11,18). Dramatic changes in the levels and properties of myocardial potassium currents have been observed with cardiac disease (8), including myocardial infarction, the death and destruction of heart muscle as a result of lack of oxygen. In canine models of myocardial infarction, potassium currents are down-regulated in cells in the infarcted zone (19-21), that is, the portion of tissue that is dying or dead due to lack of blood supply. The down-regulation is most pronounced within days following the infarct and returns to normal over the course of two months. These findings highlight the physiological importance of the regulation of potassium channels in the heart.

miRNAs regulate cardiac function

The regulation of potassium channels in normal physiology or heart disease can occur through multiple mechanisms. Transcriptional regulation plays an important role in the proper expression of different potassium channel components (8). Other mechanisms that regulate potassium channel activity include splicing, RNA editing, and post-translational modifications such as phosphorylation (8,11). In addition, microRNAs have been demonstrated as post-transcriptional regulators of potassium channel expression (2).

Mature microRNAs are small 22–26 nucleotide single-stranded endogenously encoded RNAs (22,23). Originally transcribed as pri-miRNAs, they are processed by the RNAse III *Drosha*, *Dgcr8*, and other factors to form a hairpin of ~70 nucleotides in the nucleus. The hairpins are then processed in the cytoplasm by *Dicer* to give rise to mature miRNAs (24-26). These miRNAs can associate with Argonaute proteins in complexes called RNA-induced silencing complexes (RISC). miRNAs in the RISC complex can anneal to mRNA transcripts with similar base pair sequences in their 3'UTRs or coding regions (27,28). In most cases, miRNA targeting leads to the degradation of transcripts or inhibition of their translation (29,30).

Families of miRNAs including *miR-1*, *miR-29*, *miR-15* and *miR-208* have been demonstrated to respond to cardiac stress and play a role in controlling heart function (2,8,11). Van Rooij and colleagues found that microRNAs are up- and down-regulated in cardiac tissue from mice undergoing cardiac stress (31), and in response to myocardial infarction (32). Downregulation of *miR-29*, a microRNA that targets extracellular matrix proteins (33-35), was found to contribute to the fibrotic response post myocardial infarction (32). Ikeda and colleagues extended these studies to humans, and reported reproducible changes in miRNA levels

in human patients with heart failure (36). The functional importance of miRNAs for maintaining healthy hearts has been demonstrated by cardiomyocyte-specific deletion of either *Dicer* or *Dgcr8*. Deletion of *Dicer* in 3-week-old mouse cardiomyocytes resulted in arrhythmias and lethality, while deletion of *Dicer* in the hearts of adult mice led to severe heart failure (37). Similarly, perinatal deletion of *Dgcr8* in mice resulted in severe and lethal heart failure (38).

Overexpression and loss-of function studies have revealed roles for individual miRNAs in heart disease (31,39,40). For example, *miR-208* is expressed from the intron of myosin heavy chain 6 and is expressed specifically in cardiac and slow skeletal muscle (41). *miR-208*-knockout mice respond to cardiac stress with reduced fibrosis and hypertrophy (heart cell enlargement) (42). Inhibition of *miR-208a* in rats fed a high salt diet resulted in improved survival and reduced cardiac fibrosis (43). As another example, *miR-15* is induced in the infarcted region of the heart in response to ischemia-reperfusion injury in mice and pigs (39). Systemic delivery of inhibitors of *miR-15* family members in mice with myocardial infarctions reduced the size of the infarcted region and enhanced cardiac function (39).

In addition to miRNAs that affect the survival of myocardial cells, there are also examples in which miRNAs specifically target mRNAs encoding ion channels. *miR-1* is expressed in heart and skeletal muscle and is overexpressed in individuals with coronary artery disease (44). Homozygous deletion of one of two *miR-1* genes (miR-1-2) in mice resulted in mortality of 50% of the offspring by developmental abnormalities (45). Most of the survivors died from sudden cardiac death caused by arrhythmias (45). Several targets of *miR-1* could explain these findings. In addition to the notch ligand delta (46) and the Rho GTPase *Cdc42* (47), *miR-1* also regulates Iroquois related homeobox 5 (*Irx5*), a transcriptional repressor of the I_{to} component potassium voltage-gated channel, Shal-related family, member 2 (*Kcnd2*) (45). *miR-1* also post-transcriptionally represses potassium voltage-gated channel subfamily J member 2 (*Kcnj2*), which encodes the potassium channel subunit Kir2.1, a component of the inwardly rectifying potassium channel (44), and gap junction protein alpha 1 (*Gja*), which encodes connexin 43, a connexin important for cardiac gap junctions (44). Consistent with an important role for *miR-1* in regulating action potentials, introduction of *miR-1* was found to exacerbate arrhythmias, while introduction of *miR-1* antisense inhibitors in rat hearts undergoing myocardial infarction reduced arrhythmias (44).

miR-223-3p is induced with myocardial infarction, targets Kv4.2 in its coding region, and promotes arrhythmia

In a recent issue of *Annals of Translational Medicine*: *Cellular Physiology and Biochemistry*, Liu and colleagues now report that *miR-223-3p* regulates cardiac function (48). Inspired by previous studies showing that *miR-223-3p* is upregulated in hearts after myocardial infarction (32), Liu and colleagues induced myocardial infarction in rats by tying off the artery that supplies the heart with blood (a method called left anterior descending artery ligation). This procedure resulted in arrhythmias in the rats and mortality in 40% of animals. Heart tissue surrounding the infarct area contained strongly elevated levels of *miR-223-3p* and *miR-1*.

Liu and co-authors recognized that *miR-223-3p* is complementary to, and therefore has the potential to bind to, the coding sequence of the *Kcnd2* transcript. *Kcnd2* encodes K_v4.2, the alpha subunit of the voltage-gated transient outward potassium channel that carries I_{to} in the rat during phase 1 of an action potential (48) (*Figure 2*). Consistent with this hypothesis, Liu and colleagues found that K_v4.2 protein levels were lower in the area around the rat's infarcted heart tissue. Further, down-regulation of K_v4.2 was found to be associated with reduced I_{to} flux. To determine whether K_v4.2 is a bonifide target of the *miR-223-3p* miRNA, the authors subcloned the coding sequence of K_v4.2 into a luciferase-expressing plasmid to generate a luciferase-*Kcnd2* chimeric vector, and transfected the vector into neonatal rat ventricular cardiomyocytes. Co-transfection of *miR-223-3p*, but not a negative control, substantially suppressed luciferase activity. Finally, Liu and colleagues transfected an inhibitor of *miR-223-3p* into the ventricular cardiomyocytes of rats and found that the inhibitor significantly reduced the incidence of arrhythmias after acute myocardial infarction. The findings, taken together, support an important role for *miR-233-3p* as a regulator of cardiac action potentials after a myocardial infarction in rats.

miR-223-3p inhibition as a therapy for myocardial infarction-induced arrhythmia

The findings suggest that inhibiting the induction of *miR-223-3p* during a myocardial infarction could normalize action potentials and benefit patients. Indeed, potassium channels are being recognized as important therapeutic targets and strategies to inhibit potassium channels are being

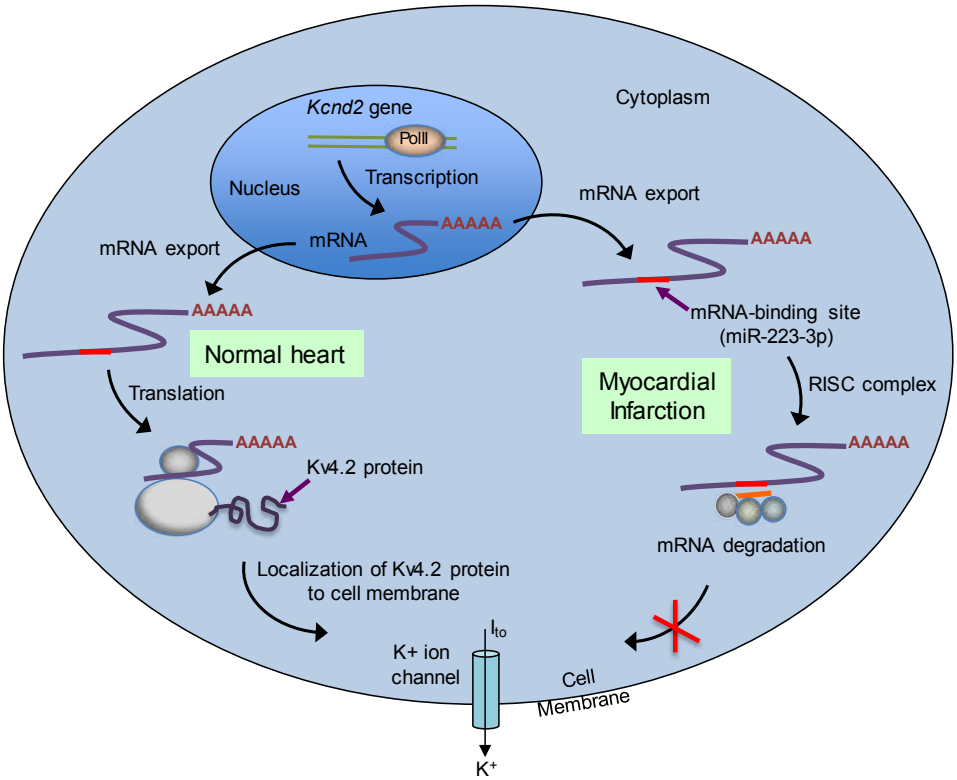

Figure 2 Schematic of the research findings of Liu *et al.* (48). In the normal heart, the *Kcnd2* gene encodes expression of the $K_v4.2$ protein that localizes to the cell membrane and forms voltage-gated potassium channels that contribute to the I_{to} current. Under conditions of myocardial infarction, *miR-223-3p* is induced and targets $K_v4.2$ for degradation through a recognition site in the coding region. Reduced levels of the voltage-gated potassium channels contribute to altered action potentials and arrhythmia after a myocardial infarction.

explored as treatment for arrhythmias and other conditions including seizures, pain, and Alzheimer's disease (49). Among the strategies to inhibit potassium channels, miRNA inhibitors are particularly appealing because they recognize specific mRNA sequence (50). Indeed, preclinical models are being developed by miRagen/Servier and other companies for the inhibition of miRNAs associated with cardiovascular disease (50,51).

The possibility of a new therapeutic strategy for myocardial infarction raises several issues. First, upregulation of *miR-223-3p* in human hearts undergoing myocardial infarction and *miR-223-3p* targeting of human *KCND2* would need to be established.

Next, *miR-223*, the same miRNA discovered to be associated with arrhythmia, has also been associated with cardioprotection. In one study, both arms of *miR-223*, 3p and 5p, were discovered to be induced after ischemia reperfusion in mouse hearts (52). But in this study, as opposed to Liu *et al.*, overexpressing a precursor miRNA containing both strands (5' and 3') of *miR-223* was associated with better contractility and reduced necrosis after myocardial ischemia (52). Transgenic mice with a knockout of the *miR-223* locus exhibited aggravated ischemia-reperfusion-induced cardiac dysfunction and more cell death (52). This study identified two cell death receptors, *Tnfr1* and *Dr6*, as *miR-223* targets in mouse hearts (52). In another study, downregulation of *miR-223* occurred in the hearts of mice with severe sepsis (53). In this study, sepsis-induced mortality, inflammation and cardiac dysfunction were exacerbated in mice with knockout of *miR-223-3p*. Semaphorin 3A was identified as a candidate gene mediating this effect. These studies raise concerns that inhibition of *miR-223-3p* in hearts would have negative consequences.

Another issue is that Liu and colleagues focus on *Kcnd2*, which is well-established as a mediator of outward potassium current in rodent heart left ventricle apex cells. However, heart rates and action potential duration differ

between species. While the phases of a heart beat are similar in all mammals, human hearts beat ~60 times per minute while rodent hearts beat ~600 times each minute (11). Until recently, the prevailing paradigm was that larger mammals such as dogs and humans rely on *KCND3*-encoded channels, while rodents use both *Kcnd2* and *Kcnd3*-encoded channels and thereby achieve more rapid depolarization required for their fast heart beats (11,54,55). However, a recent discovery of a gain-of-function point mutation in *KCND2* in a human patient with sudden cardiac arrest suggests *KCND2*-encoded proteins may be important for proper action potentials in humans as well (54). Thus, additional studies will be needed to determine the importance of K_v4.2 in human action potentials.

In addition, achieving the correct level of K_v4.2 may be challenging. Adding inhibitors of *miR-223-3p* would be expected to increase K_v4.2 and thus I_{to}. Established anti-arrhythmia drugs inhibit I_{to} (56), supporting the importance of I_{to} in establishing proper action potentials. However, inhibition of *miR-223-3p* could result in excess K_v4.2, elevated I_{to}, and arrhythmias, raising concern that miRNAs targeting K_v4.2 may increase rather than reduce arrhthyimia.

Other concerns relate to the characteristics of miRNAs. miRNAs tend to have modest effects on the target gene, off-target effects, and more pleiotropic effects than expected. For example, an anti-miR against the cardiac-specific miR-208a was unexpectedly discovered to prevent systemic phenotypes of obesity and metabolic syndrome (57). In addition, because miRNA and miRNA inhibitors are not expected to affect proteins that exist, but rather affect the levels and translation of mRNA transcripts that code for new protein, determining the pharmacokinetics of any proposed therapy will be important. Finally, developing anti-miRNAs that are not rapidly degraded and will accumulate to therapeutic levels in the heart will also be necessary (43).

In summary, proper regulation of the ion channels that control action potentials is critical for normal heart beat. Dysregulation of these channels can contribute to arrhythmia-induced mortality. miRNAs represent an emerging mechanism for regulating the expression levels of ion channel components, and are being developed as novel emerging therapeutic targets for heart disease.

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Footnote

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Circulating fibrocytes serve as a marker for clinical diagnosis

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Cardiovascular disease continues to be a major health problem in the United States and the leading cause of death (1). According to the American Heart Association, 86.5 million or more than one in three Americans have one or more cardiovascular diseases. Of the 86.5 million, 8.2 million suffer from angina pectoris. The American Heart Association predicts 43.9% of Americans will be afflicted with cardiovascular disease by 2030 (1). Despite marked progress towards the understanding of cardiovascular pathophysiology and rapid improvement in medical and surgical options, the number is still increasing. Therefore, discovery of an early diagnostic tool is important to prevent disease. Fibrocytes are progenitor cells which primarily function in response to inflammation. A recent study by Keeley *et al.* aimed to identify markers in unstable angina that may be used to predict future adverse outcomes (2). They demonstrated that the total number of fibrocytes strongly correlates with recurrent angina and unfavorable clinical events independent of risk factors. There is also evidence of expansion of circulating fibrocytes which express an activated phenotype and myofibroblast differentiation (2). These findings further support the authors' reasoning that fibrocytes have a role in vascular remodeling and their usefulness as markers. However, fibrocytes play an extensive part in immunity and utility as markers for specific pathologies may be difficult.

Identification of fibrocytes

Circulating fibrocytes are reported for first time in 1994 and are characterized as a distinct population of spindle-shaped cells with the phenotype of CD45+, collagen+, and CD34+ that are present within the blood (3). Fibrosis contributes to the pathology of a variety of diseases (4), particularly inflammatory. Due to the importance of the role of fibrocytes in tissue remodeling, much work is done to investigate the significance of fibrocyte participation in different diseases and to establish markers to detect, determine prognosis, and prevent adverse clinical outcomes. In general, mature fibrocytes have the markers CD34, CD43, CD45, LSP-1, and major histocompatibility complex (MHC) class II, which contributes to their hematopoietic nature and collagen type I and III, which explains their stromal behavior (5). Their ability to migrate to sites of injury is because they contain the markers CCR2, CCR7, and CXCR4. When fibrocytes home to sites of injury and differentiate, they change the expression of their markers. For instance, some may lose CD34 and CD45 and some may express markers to mimic the cells they specialize (5). As a result, their dynamic expression presents an obstacle to track their activity (6). Additionally, fibrocytes are derived from monocytes, thus have characteristics of hematopoietic cells and macrophages along with features of

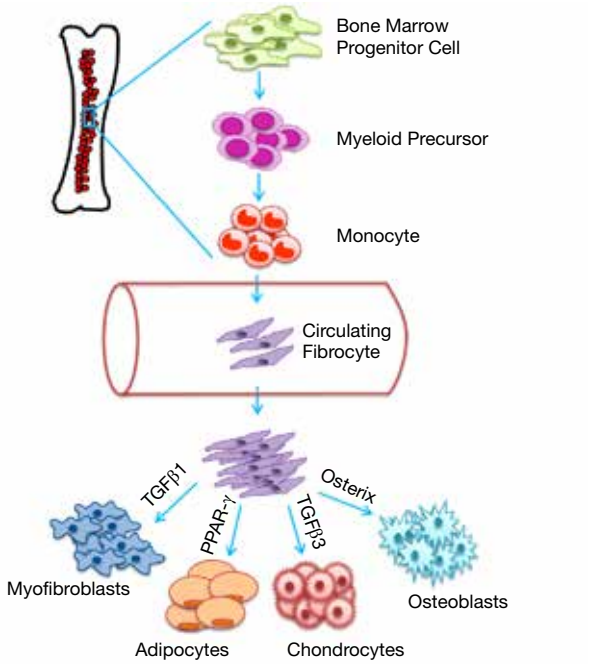

Figure 1 Generation of fibroblast and other related cells from bone marrow. Fibrocytes circulated within the blood stream and exit to migrate towards the sites of injury. These cells then differentiate into other cell types depending on the signals they receive.

fibroblasts. Therefore, finding specific markers of fibrocytes is especially arduous. Despite the challenge, one study has found that they can be distinguished from other cells because of the unique combination of CD45RO, 25F9, and S100A8/A9 expression (7). However, the discovery of more specific markers is yet to be determined.

Function of fibrocytes

Circulating fibrocytes are progenitor cells that originate from bone marrow, which circulate within the bloodstream and principally function to generate components of the extracellular matrix such as vimentin, collagen type I, and collagen type II (8,9). They are derived from monocyte precursors and have characteristics of both macrophages and fibroblasts (10). Under inflammatory conditions, these cells participate in tissue healing and repair. In response to injury, fibrocytes migrate to the inflammatory site via induction by stromal cell-derived factor 1 alpha (SDF-1α) (6). Once there, fibrocytes enhance leukocyte trafficking via increased expression of leukocyte adhesion molecules and recruitment of inflammatory cells through production of interleukin 6 (IL-6), IL-8, CC-chemokine ligand 3 (CCL3), and CCL4 (10). Repair function is initiated in fibrocytes by IL-10 and the presence of apoptotic cells (10). Additionally, neovascularization is promoted by a pro-angiogenic factor, vascular endothelial growth factor (VEGF), released by fibrocytes to aid in the repair process (6). Thus, these cells regulate immune responses via secretion of cytokines and growth factors and stimulate repair through activation of fibroblasts (11). Similar to macrophages, fibrocytes are also involved in antigen presentation to CD8+ T cells and lipid metabolism (10).

Due to the mesenchymal properties of fibrocytes, they are capable of forming myofibroblasts, osteoblasts, and adipocytes (7). The differentiation and activity of fibrocytes are primarily determined by signals in the microenvironment, which activate intracellular pathways (7). For example, a study found that transforming growth factor beta 1 (TGF-β1) increases the expression of type I collagen, alpha-smooth muscle actin (αSMA), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in fibrocytes which indicates transformation into myofibroblasts (6). Whereas, another study found that fibrocytes are under the influence of TGF-β3, which leads to upregulation of col2A1 and aggrecan and differentiation into chondrocytes (12). Fibrocytes also have the ability to differentiate into osteoblasts when stimulated by Runx2/core binding factor alpha 1 (Cbfα1) and osterix, transcription factors essential for producing bone matrix (12). Exposure of fibrocytes to peroxisome proliferator-activated receptor gamma (PPAR-γ) stimulates transformation into adipocytes (12). Direct regulation of fibrocyte differentiation has been related to CD4+ lymphocytes, which supports differentiation (7). The schematic diagram depicts the origin and differentiation of circulating fibrocytes (*Figure 1*).

Clinical implications

Studies have shown that circulating fibrocytes have a role in many cardiac diseases, particularly those involving fibrosis such as coronary heart disease, hypertensive heart disease, and cardiac ischemia (13-15). As such, markers of fibrocytes can provide valuable information regarding the extent of disease and course of treatment. In the study by Keely *et al.*, fibrocytes were examined under the condition

of unstable angina with a focus on their differentiation to myofibroblasts. The combinations of the markers CD45, αSMA, and collagen 1 were used to identify the fibrocytes and the levels of TGF-β1 to determine the breadth of expansion to myofibroblasts (2). Since both fibrocytes and myofibroblasts have general functions in healing and repair, their markers may be found in several diseases. For example, a study done in neonates with bronchopulmonary dysplasia, increased fibrocytes with αSMA has been demonstrated when compared to healthy subjects (9). However, the authors recognized that patients with known existing fibrotic diseases or other conditions may trigger fibrocyte activity and excluded them from the study.

Levels of circulating fibrocytes are relatively stable under normal conditions and rises with inflammation and hypoxic situations (13). This phenomenon can be taken advantage of in order to indirectly measure the extent of disease. Furthermore, the study by Keeley *et al.* has shown that the number of total fibrocytes correlates strongly with adverse outcomes in patients with unstable angina (2). However, the relationship is not causative and perhaps the total number of fibrocytes measures the extent of cardiac fibrosis and thus more likely to lead to unfavorable clinical events with higher numbers. Another investigation showed a positive correlation of CXCR4/procollagen-1 and CXCR4/αSMA fibrocytes with SDF-1/CXCL12^{+} expression by infarcted cells in coronary heart disease (16).

Fibrocyte markers can be applied in a clinical setting to help determine prognosis and clinical course. The ability to foresee adverse outcomes as a product of unstable angina can lead to prevention by more aggressive treatments to those at risk (2). In addition, circulating fibrocytes may be used to improve wound healing and perhaps prevent pathological fibrosis (8). More importantly, fibrocytes help determine the magnitude of fibrotic reactions (17) and may be a potential target to inhibit excessive fibrosis as seen in many inflammatory diseases. For example, class I histone deacetylase (HDAC) inhibitors regulate differentiation of fibrocytes and leads to a reduction of both fibrocytes in the heart and circulating fibrocytes when cardiac fibrosis is induced with angiotensin II (18). Moreover, fibrocytes may have the potential in regenerative medicine due to their ability to form other cell types. For instance, the capacity to form chondrocytes and osteoblasts can be used in repair, especially in damage to the articular cartilage (12). In the last few years, there have been many studies aimed to understand the importance of circulating fibrocytes in various diseases, including cardiovascular diseases. However, the currently available data suggest that circulating fibroblast might be a novel and promising therapeutic target and a marker for treatment response and prognostic evaluation.

Conclusions

Circulating fibrocytes have important biologic roles, but can also contribute to diseases related to fibrosis. Therefore, understanding these functions under normal conditions can help to prevent aberrant fibrotic processes and identification of more specific cell-surface markers may be used to predict the clinical course. The clinical importance of fibrocytes is not limited to cardiac disease, but can be virtually applied to all fibrotic diseases and may extend into regenerative medicine.

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Footnote

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Measuring soluble CD40 ligand: it is a fancy prognostic biomarker in STEMI-patients?

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Acute coronary syndromes are accompanied by progressive mechanical obstruction, dynamic obstruction, and plaque inflammation, instability, and rupture, followed by superimposed thrombosis. Myocardial ischemia and necrosis are the sequelae, followed over time by remodeling ventricular. Thus, activation of select markers and enzymes during the different phases of the process can be detected in the peripheral circulation (1).

The coronary inflammation is a primary driving force for the development and progression of atherosclerosis and, thus, increased inflammation is also a common indicator of coronary atherosclerosis. An understanding of the pathobiology of atherosclerosis and the molecular events implicated in the progression from subclinical disease to overt disease has enabled the development of biomarkers to cardiovascular diseases (2,3). The vascular wall releases molecules into the bloodstream that can reflect the pathological processes taking place there. On the other hand, blood itself is clearly involved in thrombus formation. Thus, in theory, the concentrations of the molecules involved in the different pathological processes present in atherosclerosis could be biomarkers. However, not all of these molecules are suited to this aim and should fulfil certain conditions (4,5). The characteristics of an ideal biomarker are shown in *Table 1*. Although most of the biomarkers studied up to now have been based on the possibility of being useful from the diagnostic/prognostic standpoint, it is worth recalling that ideally they would also provide a therapeutic target. Finally, although some have no diagnostic or therapeutic value, they can provide us with information on the origin and formation of atheromatous plaque (6).

Table 1 Characteristics of an ideal biomarker

Specific
Sensitive
Predictive
Fast, simple, and cheap analysis
Equal concentrations at any time of day
Samples easily acquired

Atherosclerotic plaque instability leading to adverse events is the consequence of a complex inflammatory response of the vessel wall that involves the activation of macrophages and T cells and the production of inflammatory mediators (6). Likewise, increasing evidence suggests that CD40 ligand plays an important part in disease progression and plaque destabilization (7). The CD40-CD40 ligand system is widely distributed on a variety of leukocytic and non-leukocytic cells, including endothelial and smooth muscle cell (8), and on activated platelets (9). The CD40 ligand also occurs in a soluble form that is fully active biologically termed soluble CD40 ligand (sCD40L) (10),

which are shed from stimulated lymphocytes and are actively released after platelet stimulation

The study of Napoleão *et al*. (11) was designed to identify the groups of ST segment elevation myocardial infarction (STEMI) patients with different profiles of sCD40L concentrations and verify how medication, clinical evolution, biochemical data, and markers of regulation of endothelial function at genetic (endothelial nitric oxide synthase polymorphisms) and post-transcriptional levels (circulating microRNAs) affect sCD40L serum levels. The results of this study showed that low levels of SCD40L 1 month after percutaneous coronary intervention distinguish STEMI patients with worse prognosis, a compromised cardiac healing, and a persistent endothelial dysfunction, as given by the association between genetic and post-transcriptional markers (11).

Nonetheless, the suggestion by Napoleão *et al*. (11) to encourage further studies to evaluate the clinical role of sCD40L may be overenthusiastic. Certainly, further confirmatory data from large prospective studies are needed. In everyday clinical practice, we also need simple pragmatic and practical predictors of adverse outcomes. The cardiac troponins and brain natriuretic peptide are obvious examples that are already in use. The sceptic would therefore argue that it is not only the fancy biomarker (e.g., sCD40L) that can provide clear prognostic information for death or heart failure post-acute myocardial infarction, but even very simple biomarker, such as admission troponin levels can be of value not only in assessing prognosis for cardiac events post-acute myocardial infarction but also the response to treatment. Perhaps we need to concentrate on simple things in life rather than look for too many exotic things that may be complex and expensive to measure.

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Corin as novel biomarker for myocardial infarction

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The study by Zhou *et al*. recently published in the *J Am Coll Cardiol* provides new insights into the prognostic utility of the novel biomarker corin for risk stratification of patients with acute myocardial infarction (AMI) (1). In this prospective cohort study comprising more than 1,300 patients with ST-elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI), low plasma corin concentrations were an independent predictor of major adverse cardiac events (MACE) defined as a composite endpoint of all-cause mortality, hospitalization for heart failure or recurrent AMI at a median follow-up duration of 634 days. This study impressively pointed out the incremental prognostic value of plasma corin concentrations in AMI patients beyond clinical characteristics and traditional cardiac biomarkers.

AMI is among the most frequent causes of morbidity and mortality in industrialized countries. Optimization of secondary prevention strategies is sought to be a cornerstone to improve outcome after AMI. Effective secondary prevention, again, is based on a comprehensive risk stratification of AMI patients, which preferably should be performed early after the index event. Multiple biomarkers, routinely available in clinical practice, have been linked to myocardial injury, left ventricular dysfunction and clinical outcome after AMI (2,3). However, during the last decade a number of novel biomarkers have been described, whose prognostic utility in AMI patients is either unknown or controversially discussed (4-6). As recently shown by O'Donoghue *et al*., a multimarker model including novel biomarkers could more precisely predict the occurrence of cardiovascular death or heart failure at 30 days after STEMI (7).

Corin, a transmembrane serine protease, is highly expressed in cardiomyocytes (8). Biologically, corin contributes to activation of natriuretic peptide precursor molecules and therefore plays a key role in the regulation of blood volume and blood pressure. Shedding of corin from the cardiomyocyte cell surface has been described and might reflect corin activity and cellular homeostasis (9). In patients after STEMI, plasma corin concentrations have been linked to cardiac troponin T levels and infarct size derived from cardiac magnetic resonance imaging (10). Although the impact of this study is limited due to the small sample size, these data suggest high plasma corin concentrations as an indicator of myocardial injury which is among the strongest predictors of poor outcome in AMI patients.

Now, in this imposing prospective study by Zhou *et al*. low plasma corin concentrations were related with poor clinical outcome at a median follow-up of approximately 600 days. These findings disagree with the hypothesis stated above, that intensified corin release might reflect myocardial injury (10). To a certain extent, the time point of corin measurement after AMI might explain these conflicting data. In the study performed by Zhou *et al*., corin concentrations were measured out of plasma samples collected on 'admission', which represents a rather broad time frame. On the other hand, in the study showing an association between high plasma corin concentrations and infarct size, plasma samples were collected at a median of 2 days after symptom onset. Possibly, data of the latter study

reflect corin release in the setting of AMI and therefore the extent of myocardial damage. The data shown by Zhou *et al.*, however, might reflect the widely accepted long-term deleterious effect of corin deficiency on the cardiovascular system (11). For instance, low corin expression was related with myocardial fibrosis and contractile dysfunction in a murine model of dilated cardiomyopathy (12). Contrary, overexpression of corin could improve cardiac morphology and function. In terms of these data, the study by Zhou *et al.* reflects chronic adverse effects of corin deficiency on the cardiovascular system rather than an association between corin and the extent of myocardial injury. Respectively, in this study no significant association between corin and cardiac troponin levels was observed.

In sum, the association between corin and outcome following AMI appears controversial. On the one hand, myocardial injury results in increased plasma corin concentrations suggesting intensified corin release (10). The extent of myocardial necrosis, preferably assessed using cardiac magnetic resonance imaging, is among the strongest surrogate endpoints for poor outcome after AMI (13,14). On the other hand, corin overexpression goes ahead with favourable effects on the myocardium. Hence, both mechanisms, myocardial injury and cellular overexpression, might result in increased plasma corin levels. In both cases, however, the pathophysiological agent causing corin release is completely different. The study by Zhou *et al.* suggests a predominant adverse effect of corin deficiency on prognosis after AMI. However, this study might underestimate the prognostic role of myocardial injury, particularly as cardiac troponin levels were not related with poor outcome in this study. Necessarily, larger studies are needed to further elucidate the relation of myocardial injury and corin release in the setting of AMI.

Finally, although an independent prognostic role of plasma soluble corin concentrations for poor outcome has been demonstrated in the study by Zhou *et al.*, different pathophysiological mechanisms might determine plasma corin concentrations after AMI. Studies comprising corin concentrations together with established cardiac biomarkers and imaging parameters for myocardial injury, structure and function are needed to answer this open question.

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miRNA-197 and miRNA-223 and cardiovascular death in coronary artery disease patients

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Coronary artery disease (CAD) is commonly associated with the presence of atheromatous plaques in the coronary arteries. The growth of these plaques may cause arterial stenosis and blockage of the blood flow, leading to cardiac ischemia and clinical symptoms, such as acute coronary syndrome (ACS) or stable angina pectoris (SAP). Due to the high morbidity and mortality rate, CAD has probably the most serious cardiovascular disorder threatening people's health in Western countries (1).

It is widely accepted that the erosion of vulnerable plaques results in the formation of luminal thrombi secondary to platelet activation and the release of thrombogenic elements within the atherosclerotic lesions. Indeed, coagulation components and platelet activation play a major role in the development and outcome of coronary atherosclerosis.

MicroRNAs (miRNAs) are endogenous, conserved, single stranded, small (approximately 22 nucleotides in length), non-coding RNAs that repress gene expression at the post-transcriptional level by targeting mRNA (2). According to the miRNA database (miRBase), the human genome encodes 2,588 mature miRNA sequences, which may target more than 60% of human protein-coding genes. miRNA anneals to complementary sequences in the 3'-untranslated regions (3'UTR) of target mRNAs of protein-coding genes, causing mRNA to be cleaved or to repress the translational machinery needed for protein synthesis. Thus, miRNA can either inhibit translation or induce degradation of its target mRNA or both, depending upon the overall degree of complementarily of the binding site, the number of binding sites, and the accessibility of those binding sites (3). The stronger its complimentarily with the prospective target RNA, the more likely that the miRNA will degrade the target mRNA, and those miRNAs that display imperfect sequence complementarities with target mRNAs primarily, result in translational inhibition (4,5).

Accumulating studies reveal the importance of miRNAs in regulating key signaling and lipid homeostasis pathways that alter the balance of atherosclerotic plaque progression and regression. Several miRNAs have been associated with cholesterol homeostasis by production and clearance of lipoproteins that deliver [low-density lipoprotein (LDL)] and remove [high-density lipoprotein (HDL)] cholesterol from cells and tissues. Thus, miR-148a, miR-128-1, miR-130b and miR-301b have been identified as negative regulators of LDL receptor expression and activity, promoting the clearance of circulating LDL particles (6,7). On the other hand, miRNAs have also been identified to act as critical regulators of HDL biogenesis. Many miRNAs have been identified that target ATP-binding cassette transporter-A1 (ABCA1) to reduce cholesterol efflux to apolipoprotein-A1 *in vitro*, including miR-33, miR-758, miR-26, miR-106, miR-144, as well as the above-mentioned miR-128-1 and miR-148a (8-10).

Importantly, miRNAs are not only associated with lipoprotein metabolism but they are also implied in the

regulation of endothelial cell inflammation and plaque progression. For example, several studies highlight that miR-181b and miR-146a regulate distinct components of NF-κB signaling being atheroprotective (11). Moreover, miRNAs also regulate leukocyte recruitment and activation in atherosclerosis, one of the earliest pathogenic events in atherosclerosis. A growing list of miRNAs are implicated in regulating the activation of leukocytes, including miR-let7a, miR-19a, miR-21, miR-27a, miR-33, miR-124, miR-125a, miR-146a, miR-155, miR-214, and miR-223 (12).

All these observations point out the importance of miRNAs as potential biomarkers of atherosclerosis progression and consequently, with CAD. Indeed, a number of studies have analyzed the profiling of specific miRNAs as diagnostic markers and as predictors of future cardiovascular events in CAD patients. For example, Schulte *et al*. (13) reported the capacity of miRNA-197 and miRNA-223 in predicting cardiovascular death and burden of future cardiovascular events in a large cohort of CAD patients. In this study, 873 consecutive patients [38.9% (n=340) cases of ACS and 61.1% (n=533) cases of SAP] were included in the miRNA quantification analyses after RNA isolation, and cardiovascular death was observed in 2.1% (n=18) of the patients over a median follow-up time of 4 years (IQR, 2.78–5.04). Cox regression analysis adjusted for age and gender revealed relevant prognostic power of miR-197 and miR-223 with respect to the primary end point cardiovascular death in the overall group [miRNA-197: HR =1.77 per one SD increase (95% CI, 1.20–2.60), P=0.004, C-index =0.78; miRNA-223: HR =2.23 per one SD increase (95% CI, 1.20–4.14), P=0.011, C-index =0.80]. In addition, subgroup analysis for ACS patients revealed a stronger association between elevated levels of miR-197 and miR-223 and future cardiovascular death [miRNA-197: HR =2.24 per one SD increase (95% CI, 1.25–4.01), P=0.006, C-index =0.89; miRNA-223: HR =4.94 per one SD increase (95% CI, 1.42–17.20), P=0.012, C-index =0.89].

Nonetheless, the rather small number of cardiovascular death endpoints may limit the validation of the observed findings in this study. This may influence the statistical ability to detect small effects and contains a risk of statistically overfitting the results, especially with respect to the subgroups ACS and SAP. We also need to see more characteristics of the cardiovascular death patients, as this is the primary endpoint of the AtheroGene study. Such information, together with a multivariate analysis may help the readers to clarify the role of miR-197 and miR-223 for the prediction of cardiovascular deaths in this cohort. We should stress the importance of new statistical approaches for giving us the additional information of these biomarkers in clinical practice (14).

Finally, some biomarkers for predicting cardiovascular events or deaths in community-based populations have not consistently added information to standard risk factors. Although the use of circulating biomarkers to aid risk prediction is attractive, prior studies have not consistently demonstrated the value of biomarkers for prognosis or diagnosis beyond standard risk factors in low to intermediate risk individuals in different cohorts.

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Footnote

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miR-126: a potential new key player in hypoxia and reperfusion?

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It is well known that microRNAs (miRNAs) have recently emerged as multifaceted regulators of biological processes and thus of various diseases. miRNAs are small single-stranded non-coding RNAs that bind to their cognate messenger RNAs (mRNAs) via recognition of seed sequences, i.e., the 2nd–8th nucleotide of the miRNA. This binding can trigger mRNA degradation and translational repression leading to a decrease in the protein products encoded by the corresponding mRNA. In contrast to profound effects of conventional transcription factors, individual miRNAs are rather considered as nuancing regulators of particular mRNA expression. However, since a single miRNA may act on different mRNAs and vice versa single mRNAs can be regulated by various miRNAs and additionally since miRNAs may have multiple target sites within one mRNA, cumulating effects result and complex regulatory networks are created (1).

Besides regulating translational control, circulating miRNAs act as endocrine signalling molecules and may furthermore serve as diagnostic disease markers (2). Conversely, targeting miRNA pathways offers novel therapeutic options (1).

Cardiovascular diseases remain to be among the leading causes of morbidity and mortality worldwide and impose a relevant economic burden on the health care systems. Multiple recent studies suggested a relevant role of miRNAs in cardiovascular processes and diseases.

Initially, miR-126 was demonstrated to have significant impact on tumor development and metastasis. Amongst others, it can influence inflammation, proliferation and plays a role in tumor-angiogenesis (3,4). Specifically, it seems to serve as a tumor and metastasis suppressor (3,4). In colon cancer, its expression is significantly lower especially in highly metastatic colon cancer cells. It reduces cancer cell viability and migration as well as invasion via downregulation of CXCR4 expression (3). Comparable findings have also been reported for other malignant cell lines, for example in non-small cell lung cancer via Crk (5).

More recently, a growing body of scientific findings points out the role of miR-126 in cardiovascular diseases, paralleling the earlier findings in cancer research. Endothelial cells are essential for maintaining vascular integrity, play a major role in angiogenesis and in the response to ischemia or injury (6). Vascular remodelling can be both, beneficial with repair and adaption after injury and ischemia or deleterious as for example in atherogenesis. In this context, a single miRNA can regulate multiple processes and thus affect both, positive and negative vascular remodelling (7). Due to overlappings, therapeutic targeting of miRNAs may thus influence several mechanisms in remodelling and result in unexpected and unwanted additional effects (7).

The miR-126 gene is located within an intron on the epidermal growth factor-like-domain 7 (*EGFL7*) gene

on chromosome 9 and gives rise to two mature miRNAs: miR-126-3p and miR-126-5p (6,7). miR-126 is highly expressed in the vascular endothelium and exerts distinct yet dichotomous effects in the embryonic, healthy adult and diseased adult vascular system (8). Opposing to the induction of angiogenetic signalling and promotion of endothelial cell differentiation and maturation in embryonic vasculogenesis, it preserves vascular homeostasis and integrity via inhibition of angiogenesis and endothelial cell proliferation maintaining a quiescent phenotype in the mature state (6,8-10). Besides, it is a key regulator of inflammation, which is a major contributor to vascular pathology including endothelial dysfunction, remodelling and atherosclerosis, and has effects on cells of the hematopoietic system (9,11,12). Confirmed targets include VCAM1, SPRED1 and DLK1 (7). Interestingly, the blood flow pattern and thus intravascular shear stress seem to contribute to the different effects exerted by miR-126 on endothelial cells and the vascular system (12). In the context of vessel injury and also in hypoxia, miR-126 does have differential effects. Van Solingen *et al*. showed that antagomir silencing of miR-126 did not have any effect on the proliferation and migration of HUVECs *in vitro*. Likewise, in a hindlimb ischemia model in mice induced by electrocoagulation of the femoral artery 24 h after injection of antagomir-126, no differences in blood flow recovery were seen (6). However, quantitative analysis of capillaries in the calf muscle showed a significantly lower density of capillaries in mice treated with high-dose antagomir-126. Even though miR-126 is supposed to target VCAM1 and downregulation of miR-126 might result in increased leukocyte adherence, the latter findings suggest that while not directly or at least not rate-limitingly affecting arteriogenesis, miR-126 exhibits a distinct effect on the ischemia-induced angiogenic response. In line with these findings, silencing of miR-126 impaired endothelial cell outgrowth in aortic explant cultures. A suggested mechanism for promoting angiogenesis in this setting includes a reduced expression of repressors of VEGF signalling by overexpressed miR-126 (6).

In humans, miR-126-3p was significantly downregulated in patients with acute myocardial infarction and it was suggested that administration of miR-126 could rescue endothelial cell function whereas the authors did not state the exact time of blood analysis, i.e., before or after revascularization (2).

In another analysis, an altered expression of miRNAs in patients with chronic total coronary artery occlusion and insufficient collateral arteries was recently demonstrated, with a significantly elevated level of miR-126, miR-423-5p, miR-30d and miR-10b. Even though probably suitable as biomarkers, it remains to be elucidated, however, whether these miRNAs are upregulated due to direct effects on collateral artery development or whether they are part of other pathways that affect collateral vessel growth (13). Schober *et al*. demonstrated that miR-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1 (12).

The recent *Genet Mol Res* article by Li *et al*. adds to the growing body of scientific findings the role of miR-126 in myocardial ischemia reperfusion injury. Revascularization is the gold standard for the treatment of myocardial infarction. Reviewing the current literature, Li *et al*. explain that while the exact underlying pathophysiology still needs to be elucidated injury resulting from reperfusion is a significant cause of cardiac cell death after ischemia and accounts for a relevant portion of the necrosis. Thus not only ischemia itself but also sudden blood flow restoration has a harmful effect on cell survival and apoptosis (14,15). In order to assess the role of miR-126 in reperfusion injury *in vitro*, miR-126 inhibitor and mimic were transfected into rat myocardial H9c2 cells and the cells were subjected to simulated ischemia-reperfusion injury afterwards. miR-126 as assessed by real-time PCR was significantly downregulated after ischemia-reperfusion. The miR-126 inhibitor reduced injury-induced myocardial cell apoptosis and caspase 3 protein expression. For the mimic contrary effects were observed. For *in vivo* analysis, female Wistar rats underwent lentivirus miR-126 mimic or inhibitor injection and 7 days later 30 min of myocardial ischemia induced by ligation of the left anterior descending coronary artery followed by 2 h of reperfusion. Infarction area size was significantly smaller in miR-126 inhibitor-injected animals and larger in mimic-injected animals in comparison to the control group, respectively (14). Obviously, miR-126 affects regulation of myocardial cell apoptosis in ischemia-reperfusion injury.

Contrastingly, in other investigations hypoxia itself resulted in a profound up-regulation of endothelial miR-126 (8). Furthermore, miR-126 upregulation was shown to have the capability of re-inducing angiogenesis, promoting re-endothelialization and of re-activating endothelial (progenitor) cells whilst inhibiting apoptosis and thus to contribute to healing mechanisms and to exhibit vasculoprotective effects (8). Thus, there obviously are substantial differences in the regulatory effects during hypoxia and reperfusion as well as varying effects on the

vascular system and cardiomyocytes including the involved downstream targets and mediating components. A major contributing factor in this setting might be actual blood flow and thus shear stress within the vessel. Additionally, a key role of heat shock protein 70 in influencing myocardial cell apoptosis has been suggested (14,16).

As explained earlier, miRNAs have been considered as sensitive diagnostic and prognostic biomarkers. Considering its pathophysiological implications, miR-126 may have a potential value in diagnosing not only myocardial ischemia, acute myocardial infarction, but also in stroke and other acute or chronic ischemic diseases (2,8). Additionally, the abovementioned findings underline the powerful therapeutic implications of targeting miRNAs in cardiovascular diseases. Further elucidating both conditions, hypoxia and reperfusion, could thus substantially contribute to the development of novel therapeutic agents.

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Footnote

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miR-21 alters circulating Treg function in vascular disease—hope for restoring immunoregulatory responses in atherosclerosis?

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The small molecule regulators of gene expression, microRNAs, have emerged as important mediators in a variety of cellular processes linked to disease. Much emphasis has been placed on measuring the expression of these molecules as novel biomarkers of disease. In particular, miRNA profiles of cancer have revealed both tumor specific signatures as well as highlighting common miRNAs with central roles in malignancy. Chief amongst these is microRNA-21, miR-21, the expression of which is enhanced in multiple solid tumors and lymphomas (1) where it regulates transformation by limiting the expression of various tumor suppressor genes (2). Outside of tumors, miR-21 is also highly expressed in cells of the immune system and its expression can be modulated by inflammatory stimuli during immune cell activation (3), which traffic through the circulation. miRNAs can also be found in circulation in secreted vesicles including exosomes released from various cell types associated with disease (4). This has led to an explosion in interest in measuring serum miRNA profiles during disease pathogenesis. A recently published article in *Cellular Physiology and Biochemistry* by Li and colleagues (5) measured miR-21 in serum from patients with vascular disease has revealed novel ways miR-21 can control circulating immune cell diversity and function, which impacts upon disease.

Although other groups measured circulating miR-21 in patients with atherosclerosis previously and found an increased level of miR-21 associated with increased risk of stroke (6), the study by Li and colleagues examines miR-21 in serum in various cohorts of patients with increasing severity. In particular, miR-21 expression is increased over 5-fold in peripheral blood mononuclear cells (PBMCs—a fraction of white blood cells including B, T-cells, monocytes, dendritic cells and neutrophils), in patients with severe vascular disease and a history of myocardial infarction relative to control patients with chest pains yet no vascular disease. Interestingly, in other intermediate patients groups with progressing from stable to unstable angina and increasing incidence of vascular disease there is also an increase in PBMC miR-21 expression implicating the induction of miR-21 in progression of atherosclerosis.

While atherosclerosis is associated with the recruitment of immune cells to areas of lipid deposition along blood vessels, particularly pro-inflammatory monocytes which foster and promote plaque inflammation (7), it is emerging that regulatory immune cells including FoxP3+ T-cells (Treg) infiltrate atherosclerotic plaques (8). However, the number and function of these cells decreases both in plaques and in circulation as disease progresses, consistent with a breakdown in tolerance and the appearance of pro-inflammatory T cells (9-12). The Li study confirmed this decline in circulating Treg numbers alongside the expression levels of TGF-β and FoxP3 mRNA in PBMC from atherosclerotic patients, reflected in the serum by decreased TGF-β protein levels when measured by ELISA. These decreases, like miR-21, are associated with severity of vascular disease and are more pronounced in cohorts with history of unstable angina and myocardial infarction. These

intriguing findings suggest that an increase in PBMC miR-21 as disease progresses alters T-cell function to promote an immune-regulatory environment, specifically by reducing Treg numbers and activity.

Previous work in the area of T-cell biology has implicated miR-21 in multiple levels of control of T-cell fate, function and diversity and this new data showing modulation of miR-21 negatively regulating T-reg function in atherosclerosis, while increasing our knowledge also increases the complexity whereby miR-21 can control immune function. miR-21 is known to be specifically expressed in the Treg subset (13) and promotes Treg differentiation by positively regulating expression of FoxP3 itself, the Treg-specific transcription factor. miR-21 also has the potential to positively regulate Treg activity by directly targeting a proposed negative regulator of TGF-β signaling, SMAD7 (14,15). In contrast, other studies have suggested that miR-21 in fact restrains Treg activity through intrinsic T-reg-specific mechanisms and limiting FOXP3 activity (16) or indirectly by promoting the activity of Th17 cells, whose pro-inflammatory nature counters Treg function (14). With this in mind, Li and colleagues found that the decrease in TGF-β and FoxP3 expression in PBMC from diseased patients was consistent with a decrease in mRNA expression of the miR-21 target *SMAD7*. While this suggests the increase in PBMC miR-21 has functional consequences by reducing the expression of a known miR-21 target, it also suggests the net anti-inflammatory effects of this on T-reg numbers and activity may proceed through an alternative mechanism.

Although it is likely that miR-21 controls Treg activity through repression of alternative target mRNAs, it is also possible that during atherosclerosis progression, the increase in PBMC miR-21 which alters Treg activity, may occur in other cell types which drive suppression of Tregs. In particular, the role of inflammatory monocytes, known to increase during hyperlipidemia (17), or Th17 cells, known to negatively regulate Treg activity through miR-21 in other contexts (14), could mediate the effects here. Indeed, the cells expressing induced-miR-21 in disease remain unidentified and although Treg numbers decrease with vascular disease, the possibility exists that the remaining cells enhance miR-21 to mediate suppressive effects. Although the current study did not examine miR-21 in Treg in atherosclerotic plaques, miR-21 has been reported to be up-regulated in human plaques (18) and differences in local and peripheral regulatory T-cell function may exist. The authors highlight a previous study which demonstrated that Tregs derived from umbilical cord blood (13), which represented a heterogenous population distinct from those of circulating PBMC, also express miR-21 to promote Treg activity through FoxP3.

Examining other autoimmune diseases reveals interesting differences relating to circulating miR-21 levels and immune cell function. For example, rheumatoid arthritis patients, characterized by chronic inflammation in synovial joints, display decreased serum miR-21 levels (15). This is associated with decreased Treg function and an increase in the number and activity of the Th17 subset, promoting chronic inflammation. In this particular situation, loss of miR-21 allowed expression of another predicted target, *STAT3*, which acts as a Th17-specific transcription factor. Again, the cells in which miR-21 expression is lost in during disease are not clear, although here it is likely that Th17 polarization is promoted by an increase in *STAT3* expression in T-cell precursors. This has the net effect of limiting Treg activity. In another model of autoimmune disease, the EAE model of multiple sclerosis, targeting miR-21 using antisense was shown to block Th17 mediated inflammation leading to reduced disease burden (14). In mouse models of SLE, both targeting of miR-21 by antisense and miR-21 deletion decreased Th17 cells with a consequent increase in Tregs, conferring protection against disease (19,20). These results confirm that the miR-21/Treg axis is active in disease and confirmation of the results in the Li *et al.* study using similar tools for vascular disease are forthcoming. In particular, identifying the mRNA targets specific to each disease through which miR-21 has its effects on immune cell function will add to the complexity behind control of T-cell fate by this important miRNA and will allow more specific targeting for improved therapies.

In summary, the study by Li *et al.* provides primary patient data highlighting an important role for miR-21 in the pathogenesis of vascular disease. Notably, as disease progresses there is a loss of an important immunoregulatory T-cell subset in circulation which is driven by miR-21 and identifying the cellular and molecular mechanisms controlling this will illuminate our knowledge of T-cell function, tolerance and inflammation in atherosclerosis. In particular, identifying the signals, such as lipids or lipid-induced inflammatory mediators, which drive miR-21 expression during disease progression as well as identifying the overexpressing subsets in PBMCs and correlating these observed differences with local immune cells in the plaque, will improve our understanding of miR-21 function in immunity.

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The role of circulating microRNAs in acute coronary syndromes: ready for prime time?

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Recently Navickas *et al*. published a review on the role of microRNAs (miRs) as biomarkers of cardiovascular disease in *Cardiovascular Research* (1). Based on a systematic literature research their aim was to determine the diagnostic and prognostic value of miRs in healthy subjects, subjects with stable coronary artery disease and patients with different forms of unstable coronary artery disease (unstable angina, non-STEMI and STEMI). They identified n=487 papers and extracted n=19 studies, reporting on 52 different miRs, after a rigorous quality check. The largest amount of evidence, through all stages of cardiovascular disease, was found for miR-133a/b (5 studies), miR-208a/b (6 studies) and miR-499 (7 studies). Furthermore the promising role of miR-1 (3 studies) in the diagnosis of acute coronary syndromes and the regulation of miR-145 in STEMI patients is highlighted. A meta-analysis, however, is not presented because of heterogeneous study designs and analytical reasons (1).

Especially in patients with acute coronary syndromes the role of novel biomarkers is rapidly evolving (2). Cardiac troponin measured with standard (3) and high-sensitive (4) assays has improved our abilities to define patients with acute coronary syndromes (5), estimate the amount of myocardial necrosis (3), predict functional impairment (6,7) and prognosis (8). Another well-established biomarker in acute coronary syndromes is NT-pro-BNP (9). Today these two biomarkers impact clinical decisions of cardiologist every day. However their diagnostic performance is hampered by diagnostic windows, their relatively low specificity (10) and their correlation with renal function (11). Therefor there is indeed a need to identify novel biomarkers in acute coronary syndromes.

miR-1 is very specific for cardiac skeletal muscle and plays an important role during cardiogenesis and proliferation of cardiomyocytes (12). Three different studies, with a total of n=583 patients with acute coronary syndromes and n=259 controls (13-15), investigated the role of miR-1 in the initial diagnosis of patients with chest pain and suspected acute coronary syndrome. Wang *et al*. observed that miR-1 levels in patients with acute myocardial infarction are elevated compared to controls, but the diagnostic performance of miR-1 was inferior to cardiac troponin I (AUC: 0.85 *vs*. 0.99) (13). Oerlemans *et al*. described an increase in miR-1 even in patients with initially negative troponin levels or in patients presenting within 3 hours after symptom onset (14). Furthermore, Widera *et al*. showed that miR-1 levels are significantly higher in patients with NSTEMI or STEMI than in patients with unstable angina, although they did not predict mortality at 6 months (15). Anyhow, these results identify miR-1 as one of the most promising miRs for the early diagnosis of acute coronary syndromes, especially in the combination with other biomarkers.

The largest study included in this review was performed by Devaux *et al*. (16). It prospectively investigated the use of six different miRs in n=1,155 patients with acute chest pain and suspected acute myocardial infarction. Finally,

n=179 patients were diagnosed as NSTEMI and n=45 patients as suffering from a STEMI. miR-133a, miR-208b and miR-499 were identified as univariate predictors of myocardial infarction. As noted by Navickas *et al*. all these three miRs control cardiomyocyte identity (17). However, their predictive value did not remain significant after correction for troponin levels. Furthermore, the area under the receiver operating curves were low (AUC: 0.53–0.76) compared to high sensitive troponin (AUC: 0.94). The miR-133a, miR-208b and miR-499 levels were significantly higher in STEMI patients than in NSTEMI patients (16). These findings are in line with the findings of Widera *et al*. on miR-133a and miR-208a (15), although both studies failed to demonstrate an independent prognostic value of all miRs studied.

The potential unique role of miR-133a in STEMI patients is further highlighted by a study by Eitel *et al*. (18). In this study miR-133a levels were associated with decreased myocardial salvage, larger infarct size and microvascular obstruction (19) as determined by cardiac magnetic resonance in a clearly defined study population of n=216 consecutive STEMI patients. Although miR-133a was a univariate predictor of mortality and MACE (HR: 1.28) the use of cardiac magnetic resonance for infarct characterisation (20) allowed the authors to demonstrate, that this association is not independent of infarct characteristics (18).

Another study focusing exclusively on patients with STEMI was performed by Dong *et al*. (21) who investigated the prognostic value of miR-145, which regulates vascular smooth muscle cell and cardiomyocyte differentiation and has been shown to correlate with infarct size (22). In n=245 with STEMI they demonstrated that miR-145 levels above the median predicted 12-months MACE independent of NT-pro-BNP, creatine kinase or troponin levels (HR: 5.6) (21). Interestingly miR-145 levels have been observed to be generally lower in patients with severe coronary artery disease or acute coronary syndromes which might indicate altered expression of miR-145 in these patients (23). As these findings seem controversial further research should clarify the role of miR-145 in cardiovascular disease.

Navickas *et al*. have done a valuable work in identifying five, out of more than 2,000 described in humans, miRs which have great potential to improve our daily clinical work in the future. Their review is based on the data of 19 studies with more than 6,000 participants (1). These miRs are ready for prime time in cardiovascular research but further studies are warranted to provide reliable and standardised quantification with faster PCR and microarray technologies. Then it should be possible to include these promising biomarkers in controlled, large-scale, well-powered trials and, perhaps someday, into clinical practice.

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Should we expect novel biomarkers of myocardial infarction?

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Recent studies have revealed important roles for microRNAs (miRNAs) in cardiovascular disease, including acute myocardial infarction (AMI). miRNAs are small, 20–25 nucleotides long, non-coding RNA molecules, which inhibit gene expression by promoting mRNA degradation or preventing translation (1,2). Although the biological functions of miRNAs are not fully understood, numerous studies have shown that some miRNAs have unique expression profiles in certain tissues or cell types (3). Recent discoveries have revealed the existence of freely circulating, stably expressed miRNAs in human blood cells or plasma/serum (4,5). The circulating miRNAs have been shown to be sensitive and informative biomarkers in the diagnosis of cardiovascular diseases. The article by Yao and colleagues (6) describes circulating miRNA-122-5p as a potential novel biomarker for diagnosis of acute myocardial infarction. In that study, the authors investigated the level of miRNA-122-5p by quantitative real-time PCR (RT-qPCR) and found that its expression was up-regulated at 4, 8, 12, and 24 h in AMI patients compared to non-AMI controls, and displayed similar trends to the cTnI concentrations. A high correlation was observed between the circulating miR-122-5p and cTnI concentrations. The receiver operating characteristic (ROC) curve analysis showed that miRNA-122-5p in plasma had considerable diagnostic accuracy for AMI with an area under curve (AUC) of 0.855. The results suggest that miRNA-122-5p could leak from cardiac myocytes into the circulation during the early stages of AMI.

This circulating miR-122-5p could be a useful biomarker for the diagnosis of AMI. Furthermore, miRNA expression analysis, particularly when combined with clinical parameters, such as cTnI concentrations, provides better understanding of the changes that occur in the myocardium and determine the potential role of extracellular miRNA-122-5p as a paracrine signaling molecule. The authors took into account that the small sample size was a major limitation of the study.

The authors do not state how they found out that this miR-122-5p might be elevated in AMI—until now it was mainly linked with liver injury (7). However, some papers showing upregulation of miR-122 (miR-122 and miR-122-5p are originating from the same hairpin, see e.g., https://www.exiqon.com/mirsearch) in AMI have also been published (8,9). Of note, the study by Li *et al.* (9) was performed using miRNA microarrays representing 1,205 human miRNAs and the results were validated on a large group of patients (two independent cohorts of 111 and 428 patients). Therefore, we have another replication showing increase in miR-122-5p expression in patients with AMI.

Interestingly, numerous studies have indicated that heart-specific miRNAs could be released into the circulation during AMI, making them potentially useful in aiding diagnosis or guiding therapy in acute coronary syndrome (10). Such miRNAs can easily be detected in the circulation and serve as potential biomarkers for cardiovascular diseases. For instance, Gidlöf *et al.* (11) found that the plasma level of some cardiac-associated miRNAs, such as miRNA-1, miRNA-133a, miRNA-208b, and miRNA-499-5p significantly increased in STEMI patients. D'Alessandra *et al.* (12)

have reported that in an acute hind-limb ischemia, unlike in AMI, plasma levels of miRNA-1, miRNA-133a, miRNA-133b, and miRNA-499-5p did not increase, indicating that they are ideal biomarkers for AMI. Ai *et al*. (13) showed that circulating miRNA-1 level was significantly higher in AMI patients compared with non-AMI group and the level returned to normal on discharge following medication. Corsten *et al*. (14) evaluated plasma levels of heart-associated miRNAs (miR-1, miR-133a, miR-208b, and miR-499), fibrosis-associated miRNAs (miRNA-21 and miRNA-29b), and leukocyte-associated miRNAs (miRNA-146, miRNA-155, and miRNA-223) in patients with various cardiac damage including AMI, viral myocarditis, diastolic dysfunction, and acute HF. miR-208b and miRNA-499 were found to be significantly elevated in AMI patients compared to healthy controls. The ROC curve analysis revealed a good diagnostic value of miRNA-208b and miRNA-499 as biomarkers for AMI.

Circulating miRNA-122-5p could broaden our understanding of the role of miRNAs in the pathogenesis of acute coronary syndromes and, if confirmed, it could become a novel biomarker for AMI. We need publications on large cohorts of patients with clinical follow-up, to see if expression of miR-122-5p gives us additional predictive value to clinical data and classical biomarkers. The real revolution is just behind the doorstep—first attempts are currently being made to use miR antagonists for the treatment of patients: now those with hepatitis C, but a novel field in cardiology is opening up (15).

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Footnote

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The hunt for fatal myocardial infarction biomarkers: predictive circulating microRNAs

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Introduction

The concept of a hype cycle is a well-established business concept, in which novel ideas are said to have an initial wave of hype followed by disillusionment. Only after that, the novel concept takes off and become truly useful entering a so-called plateau of productivity. In biomedical science, the field of microRNAs (miRNAs) certainly had a peak of interest in the end of the last decade. This led by high impact publications (1) and characterization of both novel miRNA-entities as well as their associations to a broad range of diseases. Nonetheless, no clear pharmaceutical successes emerged: miRNA targets are being pursued as therapeutic targets, but none have as of yet successfully made it through clinical trials (2). Likewise the use of miRNA-based treatment strategies targeting regular mRNA is an area of interest (3). In this editorial we focus on a third aspect of miRNAs: the use of miRNAs as prognostic biomarkers in disease, asking the question if miRNAs are now entering this plateau of productivity in which actual benefit will be seen.

We focus on the recent paper by Bye *et al.*: "*Circulating microRNAs predict future fatal myocardial infarction in healthy individuals - The HUNT study*" (4). This paper is of particular interest because it presents strong evidence for prognostic benefit of miRNAs. The study was based on the HUNT cohort, a Norwegian biobank-initiative in which an impressive 88% of the adult population of the Nord-Trøndelag County participated, giving blood samples and questionnaire information in 1984, 1995, and 2006. With the unique advantage of having both frozen serum and decades of follow-up information, the study was designed as a prospective nested case-control design with fatal acute myocardial infarct (AMI) as endpoint and controls matched on risk factors such as body mass index (BMI), total cholesterol and high-density lipoprotein cholesterol (HDL-C) (4). The main discovery phase results yielded 12 miRNAs that were associated with future AMI. This editorial will discuss the perspectives of these findings as well as considerations for similar future miRNA studies.

The methodology of miRNA normalization

A key consideration in biomarker studies is the existence of similar studies. For miRNA biomarkers predicting AMI, several studies already exist that address AMI risk. Typically the main focus is the discovery of biomarkers for immediate use, such as distinguishing patients with ST-elevated AMI from patients with stable ischemic heart disease (5), or between ongoing AMI and healthy controls (6). The most comparable study to the interest of this editorial is the one Zampetaki *et al.* In this study,

the main focus was the prediction of future AMI and the authors did find association of miR-126, miR-223 and miR-197 to the disease (7). It is noteworthy that none of these miRNAs were identified by Bye *et al.* (4). Hence, for the overall purpose of using miRNAs as predictive biomarkers it prompts an important discussion on methodological choice.

Bye *et al.* suggested that one main discrepancy reason could be the choice of data normalization method and the platform for miRNA analysis. In fact, while Bye *et al.* used a panel of seven reference genes for normalization and quality control by means of the RNA Spike-in kit including cel-miR-39-3p, UniSp2, UniSp4, UniSp5 and UniSp6, Zampetaki *et al.* (7) solely used U6, which is not a suitable endogenous control for the quantification of circulating miRNAs based on previous works (8,9). It has been shown that the Spike-in system improves the quality of the normalization step (10). The normalization method for circulating miRNA quantification is one of the critical aspects in this field and from this point of view the normalization procedure used in the work of Bye *et al.* is the most robust to date.

Sample collection and processing in miRNA analysis

Another crucial aspect for the analysis of circulating miRNAs is the collection and processing of blood samples. In fact, it has been suggested that blood must be processed within a few hours after collection in order to prevent cell-derived miRNA contamination from red blood cells or platelets (11-13). Unfortunately, many studies do not follow this suggestion or do not report this important information. Moreover, it has been shown that the difference between serum and plasma strongly affects the spectrum of circulating miRNA in blood (11) demonstrating higher miRNA concentrations in serum samples compared to the corresponding plasma samples. Considering that Bye *et al.* and Zampetaki *et al.* extracted miRNAs from serum and plasma, respectively, and that they used two different RNA isolation kits (miRCURY RNA isolation and miRNeasy kit, respectively), it is plausible that the two studies did not find the same miRNAs. All these considerations point the attention on the fact that, given the numerous factors that generate variability in circulating miRNA studies, it is now mandatory to develop standard protocols for blood specimen collection and processing to allow the comparison across studies.

Using small RNA-seq to improve the quality of the results

Circulating miRNAs are considered novel non-invasive biomarkers. Yet, the mechanism of action at the molecular level both in healthy and disease is still largely unknown. Since there is a great opportunity to establish a new paradigm of intercellular communication, the National Institutes of Health (NIH) funded a novel Common Fund's Extracellular RNA Communication (ERC) program "(I) to discover fundamental biological principles about the mechanisms of extracellular RNA (exRNA) generation, secretion, and transport; (II) to identify and develop a catalogue of exRNA in normal human body fluids; (III) and to investigate the potential for using exRNAs as therapeutic molecules or biomarkers of disease". In order to disseminate the knowledge derived from this program, the results are shared through the exRNA research portal, a community-wide resource for exRNA standards, protocols and data. These efforts have already generated new small RNA-seq data for several conditions (including cardiovascular diseases and cancer), biofluids (e.g., plasma and serum) and RNA sources (e.g., exosomes and other extracellular vesicles). Since the quality, the amount and the specific body fluid are important factors (as discussed above), RNA-seq is likely to be the future standard technique in this field. Still, small RNA-seq is not the common method used as shown in the miRandola database, the circulating RNAs database (14). In the work of Bye *et al.* and in many other published studies, qRT-PCR has been used as golden standard for miRNA quantification. Since in this context the normalization step is crucial and there is no clear agreement in the scientific society, using small RNA-seq could solve this crucial problem, increasing the quality of the results. Overall, rigorous standardized methods and analyses are needed in this field. It has been reported that many confounding factors exist in the phases of processing, extraction and quantification of exRNAs.

Statistical considerations in search for predictive biomarkers

In understanding biomarker discovery studies it is important to be very aware of the statistical pitfalls associated with them (15). The archetypical discovery study pitfall is that of the winner's curse; that testing hundreds of metrics will inevitable yield significant findings by chance and that their effect estimates will be inflated (16). In the Bye *et al.* study (4)

the study design was built around a discovery phase as well as a validation phase. In the discovery phase, 76 miRNAs were tested, of which 12 were selected at an uncorrected $P<0.05$ significance threshold. This alone is of course a clear example of test metric inflation and winner's curse, and it follows that the $\Delta\Delta$Cq values should decrease in the validation phase, which in fact they do. However, the study also included a validation phase within which it was shown that ten of the 12 miRNAs were significantly associated with future AMI at $P<0.05$. No metric was provided reporting with a formal multiple testing correction of 12 miRNAs, but it is reported that four of the 12 miRNAs also were significant at $P<0.01$. Further, Bye *et al*. presents a combination signature of five other miRNAs. These resulted from the testing of 4,095 different combinations with no independent replication. It is from this signature that the 0.91 AUC is concluded.

The replication and validation setup is a good strategy to amend winners curse problems. Ultimately, however, the proper question to debate here is of course whether these statistics will hold up in a general case. The combination signature score of 0.91 AUC finding was based on a large un-replicated multiple burden and so is highly likely to decrease on independent validation. However, the individual miRNA scores were independently replicated suggesting that they are true in a general case. And that is a novelty in a field burdened by underpowered discovery studies without genuine follow up, so we believe that at least the four strongly replicating miRNAs, let-7g-5p, miR-26a-5p, miR-106a-5p, and miR-151a-5p, could play important future roles in the field of AMI prognostics.

Conclusions

Current prediction algorithms in clinical use include the European Society of Cardiology's Systematic Coronary Risk Evaluation (SCORE) and the Framingham risk score (17,18). These algorithms have some impact in a clinical setting; by accurately assigning patients to risk-groups they can prompt important discussions of smoking patterns, LDL-cholesterol levels and overall healthy lifestyle. However, the scores are still too inaccurate to clearly pinpoint the individuals who will in fact become future patients. This is the reason why predictive biomarkers are of such interest. Having the ability to identify individuals who has a high risk of adverse events with only low chance of false positives; that is a hallmark of precision medicine, and one that is not possible only with the current life-style based clinical scores.

While the work with miRNA biomarkers for AMI is still in its infancy, studies like Bye *et al*. pave a way for a future in which life-style scores could be supplemented with simple and cheap blood-test-based biomarker predictions, and resultant in early and accurate intervention. More accurate intervention, importantly, also means less wasteful and non-required treatment of individuals who are in fact not at risk. And this optimization of the health care system really is the grand perspective to have in mind when considering precision medicine in general, and studies like Bye *et al*. in particular.

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Footnote

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Correlations between microRNAs and their target genes in skeletal myoblasts cell therapy for myocardial infarction

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In the setting of ischemic heart disease in which revascularization therapies (both percutaneous and surgery) and pharmaceutical therapy are able to contribute to ventricular remodeling, nowadays we also have some indications about promising options to prevent or reverse the ventricular remodelling process and consequent heart failure. Stem cells provide an alternative curative intervention for the infarcted heart by compensating for the cardiomyocyte loss subsequent to myocardial injury. The presence of resident stem and progenitor cell populations in the heart, and nuclear reprogramming of somatic cells with genetic induction of pluripotency markers are the emerging new developments in stem cell-based regenerative medicine. However, until safety and feasibility of these cells are established by extensive experimentation in *in vitro* and *in vivo* experimental models, skeletal muscle-derived myoblasts, and bone marrow cells remain the most well-studied donor cell types for myocardial regeneration and repair (1,2). Furthermore in the literature we can find a lot of indications about microRNAs as novel and alternative cardiac biomarkers. These are 22-nucleotide-long non-coding RNAs that regulate gene expression at post-transcriptional level. Several recent studies have shown that miRNAs play a physiological role in cardiovascular homeostasis and in the pathogenesis of cardiovascular disease. Expression-pattern studies of myocardial tissue reveal that several miRNAs are up- or down-regulated during myocardial infarction (3,4).

During nearly two decades of cell therapy research for treatment of ischemic heart disease, stem cell transplantation, either alone or in combination with the other therapeutic interventions, has demonstrated promise as a novel curative strategy (5). The prime advantage of the heart cell therapy using stem cells is its capability to replace the loss-of-functioning cardiomyocytes to preserve the deterioration of left ventricular function (6,7). Of all stem cells used, skeletal muscle-derived myoblasts have been investigated in experimental and clinical studies; in fact skeletal myoblasts and bone marrow derived stem cells remain the most well-characterized studied therapy for myocardial reparability in the patients with ischemic heart disease (8,9). Skeletal myoblasts constitute the renewable source of progenitor cells in skeletal muscle that participate in the repair process in the event of injury. The most important characteristics of skeletal myoblasts that make them suitable for use are their autologous availability, potential to expand *in vitro*, resistance to ischemia, low risk of tumorigenesis and myogenic differentiation potential (10,11). In the paper of Liu *et al.*, published on *Journal of Transplantation Medicine* in 2015, the authors analyzed the correlations between microRNAs and their target genes in skeletal myoblasts cell therapy for myocardial infarction. Their final data showed that the down regulation of apoptosis-regulatory microRNAs and in turn up regulation of target genes may partially account for rescue effect of skeletal myoblasts

therapy for myocardial infarction (12). Since the early 2000s we can find in the literature a lot of *in vitro* researches that have assessed the performance of skeletal myoblasts for the treatment of ischemic and non ischemic cardiomyopathies in animal models (13-16). All these studies showed that skeletal myoblasts prevented left ventricular remodeling, preserved ejection fraction, left ventricular pressure wall thickness and left ventricular pressure. Skeletal myoblasts were, also, used in post myocardial patients in some clinical settings (17,18). Even in these cases, results from heart function were promising increasing in left ventricular ejection fraction and in segmental contractility on echocardiography. The interesting feature of the paper of Liu *et al*. (12) was to compare the expression of microRNAs in post myocardial infarction rats with or without skeletal myoblasts cell transplantation. The authors focused their research on new apoptosis-associated microRNAs and their target genes; in particular they showed that four microRNAs were down regulated in the skeletal myoblasts treated group compared with the untreated group. Some studies in the recent past showed the role of microRNAs; some of this have distinct roles in modulating skeletal and cardiac muscle proliferation and differentiation (19-21); other studies have, also, demonstrated that microRNA-206 and -1 directly down regulated gap junction coupling after the initiation of myoblast fusion *in vitro* and *in vivo* and inhibit Cx 43 expression during myoblast differentiation without altering Cx43 microRNA levels (22). The paper of Liu *et al*., (12) is the first comparative *in vitro* study of microRNA and microRNA expression in myocardial infarction heart treated with skeletal myoblasts transplantation using surgical route. We hope as interesting for the future skeletal myoblasts transplantation combined with transplantation of other cell types or growth factor; a combined therapy based on simultaneous delivery of skeletal myoblasts and bone marrow stem cells may be more effective with either cell type alone as partially demonstrated in the recent past (23). In-depth mechanistic studies, genetic reprogramming or pharmacological manipulation, and combinatorial approach involving skeletal myoblast transplantation with other relevant interventions, such as growth factor administration, can help safety and therapeutic efficacy; in fact simultaneous insertion of skeletal myoblasts with multiple growth factors promoted their ability to integrate with host myocytes. Skeletal myoblasts remain the most well-studied donor cell type.

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Footnote

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Is the regulation of SIRT1 by miRNA-34a the key to mesenchymal stem cell survival?

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Mesenchymal stem cells (MSCs) are currently being used clinically to treat cardiovascular diseases, including ischemic heart disease, heart failure, and peripheral vascular disease (1,2). To date, these trials have proven MSCs to be a safe and effective option for the improvement of vascular function, reduction of scar size, and reversal of remodeling in heart failure (3,4). Despite the positive data being collected, there are still a number of challenges that reduce the effectiveness of MSC cell therapy. Cell survival and engraftment in the hostile microenvironment of the diseased myocardium severely limits MSC regenerative potential. Research focusing on MSC biology, including the identification of genes and molecules that regulate aspects of regeneration, is critical towards the establishment of effective cell production and delivery strategies (5,6).

Gene therapy and the manipulation of protein expression via genetic editing and targeted microRNA (miRNA) technologies has become a promising avenue for the enhancement of cellular regenerative potential (7). The delivery of genetically modified MSCs to ischemic hearts in pre-clinical studies has advanced our understanding of which genes could potentially improve the efficacy of MSC therapy. Overexpression of genes such as extracellular superoxide dismutase (ecSOD) and vascular endothelial growth factor (VEGF) in MSCs has improved survival and tissue-repair when cells are injected into sites of ischemic injury (8,9). Similarly, modification of miRNAs via lentiviral induced expression or silencing has made it possible to target entire pathways rather than individual genes. Delivery of MSCs transduced to overexpress miRNA-126 enhanced ischemic angiogenesis through the activation of AKT and ERK regulated signaling pathways (10). Together, these studies have provoked further investigation to establish the optimal gene targeting systems that will optimize MSC therapeutic applications.

In an intriguing study published in *Stem Cell Research and Therapy*, Zhang *et al.* demonstrated a novel role for miRNA-34a in the regulation of apoptosis and senescence by MSCs via the silent information regulator 1 (SIRT1)-mediated pathways (11). As a well-studied miRNA, the down-regulation of miRNA-34a promotes proliferation, increases stress resistance, and promotes cell migration in a variety of cell types. These outcomes are particularly appealing to a cell-therapy application where cell survival and tissue integration is at risk. The therapeutic advantages of modifying miRNA-34a expression are further showcased through the feasibility of specific alteration using oligonucleotide mimetic or inhibition. Particularly in cancer therapy, induced expression of miRNA-34a safely reduced tumor growth and survival (12). In this context, the authors describe that MSC apoptosis and decline of cell proliferation correlates with miRNA-34a upregulation. This response serves as a promising therapeutic target in MSCs

because recent studies have demonstrated a consistent role of miRNA-34a in cell cycle progression, particularly due to its regulation of genes such as p53, c-kit, SIRT1, and Notch (13). Based on these findings, the authors formulated and tested the hypothesis that overexpression of miRNA-34a exacerbates hypoxia and serum starvation-induced apoptosis and senescence in MSCs.

The combination of hypoxia and serum starvation is a common inducer of MSC apoptosis, and is a reliable model to test molecular mechanisms that may be altered upon cell injection into hypoxic tissue (14). Zhang *et al.* used this model to test the potency of miRNA-34a overexpression and silencing on cell apoptosis via the detection of annexin-V positive cells. As expected, those MSCs engineered to overexpress miRNA-34a exhibited a greater amount of apoptosis upon hypoxic serum starvation conditions, while silencing miRNA-34a significantly reduced this effect. These findings were then expanded upon in order to identify the key molecular players involved in the miRNA-34a response.

SIRT1 is an established and key target of miRNA-34a due to its endogenous regulation of anti-apoptotic and pro-proliferative pathways. Acting as a NAD-dependent protein deacetylase, SIRT1 has been described as a longevity factor, targeting transcription factors such as forkhead box proteins (FOX) and p53, while tightly regulating cellular resistance to oxidative damage (15). However, it had not been previously shown whether miRNA-34a manipulation and subsequent regulation of SIRT1 affects MSC biology. The authors used SIRT1 gene silencing paired with miRNA-34a inhibition to test the involvement of SIRT1 in the miRNA-34a-mediated apoptotic response. The authors demonstrated that miRNA-34a regulates SIRT1 and the downstream pro-apoptotic factor FOXO3a. Moreover, silencing SIRT1 expression abolished the anti-apoptotic effects of inhibiting miRNA-34a. This result was confirmed by the alteration of apoptotic markers caspase-3, poly ADP ribose polymerase (PARP), and mitochondrial cytochrome C. Lastly, markers of DNA damage and senescence decreased with miRNA-34a inhibition, providing further evidence for the multifaceted effects of miRNA-34a in MSCs.

Although these findings serve as a promising first step toward establishing a genetic strategy designed to effectively preserve the viability of MSCs delivered into the ischemic myocardium, many questions are still left to be resolved. The broad range of RNA targets of miRNA-34a may be beneficial or problematic, and the long-term effect of miRNA-34a inhibition on MSCs has not been explored. The goal of MSC therapy is for transplanted MSCs to provide lasting reparative effects on myocardial structure and function by engrafting into the myocardium. miRNA-34a overexpression vectors are currently being investigated in translational models of cancer to halt unregulated cell proliferation and cancer metastasis (16). Whether the permanent inhibition of miRNA-34a in MSCs is a safe option that does not pose tumorigenic risk needs to be determined. Additionally, genes that influence MSC differentiation and angiogenesis, two important factors for the regenerative response, are known targets of miRNA-34a. Expression of platelet-derived growth factor receptor (PDGFR), which is down regulated by miRNA-34a, plays a significant role in MSC mediated vasculogenesis (17,18). It would be important to investigate how the regulation of multiple pathways by miRNA-34a would impact the *in vivo* differentiation and tissue integration of MSCs. Lastly, the experiments performed by Zhang *et al.* were all done in 5% oxygen (11), a level that is hypoxic compared to standard laboratory culture conditions (21% oxygen) but not compared to the physiological oxygen levels in the mammalian heart. It has been reported that ischemia in the heart could decrease oxygen levels from a physiologic 5% to a low of 1–3% oxygen (19). To confirm the effectiveness of miRNA-34a silencing in ischemia, similar experiments would need to be conducted at lower oxygen tensions.

In summary, Zhang *et al.* have presented a novel and promising genetic targeting strategy that could enhance the effectiveness of MSC therapy in ischemic heart disease. Their findings demonstrate that miRNA-34a inhibition results in greater viability of MSCs in an *in vitro* model of hypoxia and serum starvation, warranting further investigation in an *in vivo* translational model.

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Footnote

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My heart will go on—beneficial effects of anti-MiR-30 after myocardial infarction

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Abstract: MicroRNAs play key roles in the regulation of diverse cellular processes and during cardiovascular disease development and progression, such as myocardial infarction (MI) and heart failure (HF). A recent manuscript by Shen and colleagues provided evidence that the miR-30-CSE-H_2S axis contributes to the protection against cardiomyocyte ischemic injury by regulating hydrogen sulfide (H_2S) production. Inhibition of the miR-30 family after MI injury offers potential therapeutic value to 'keep our heart going on'. As this study highlights miRNAs as promising future therapeutic targets, their translational applicability to utilization in humans needs to be viewed with caution.

Keywords: microRNAs; myocardial Infarction (MI); therapeutics; cardiomyocytes

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MicroRNAs (miRNAs) are small, non-coding RNA molecules, approximately 22 nucleotides in length, which act as master regulators of gene expression (1). Mature miRNAs are single stranded RNAs that block translation—or induce degradation of mRNA by base pairing to partially or perfectly complementary sites on their target mRNA, usually in the 3'-UTR (2). Accumulated data have indicated that miRNAs play key roles in the regulation of diverse cellular processes and during cardiovascular disease development and progression, such as atherosclerosis, hypertension, arrhythmias, left ventricular hypertrophy, myocardial infarction (MI), as well as heart failure (HF) (3,4).

Despite all available therapeutic approaches, MI still ranks as the main cause of death worldwide (5). Pathophysiological landmarks of MI include acute myocardial damage, post-ischemic neovascularization, and cardiac remodeling (6). Acute myocardial damage is attributed by ischemic cellular hypoxia, which results in an increase of reactive oxygen species during early reperfusion, endothelial cell (EC) activation, cytokine production in the damaged area, priming and recruiting of neutrophils bordering the infarcted region, and ultimately cardiomyocyte death, endothelial capillary impairment and post-ischemic neovascularization. The remodeling process is initially an adaptive mechanism to maintain adequate cardiac function, but eventually leads to fibrosis, left ventricular dilatation, and HF (7).

Evidentially, all these processes are tightly regulated by miRNAs (6). Primarily, miRNAs participate in the regulation of cardiomyocyte fate, such as the pro-apoptotic miRs-15, -34, -320, and -140; the anti-apoptotic miRs-24 and -214; the pro-proliferative miR-17–92 cluster, miRs-199a and -590; and anti-proliferative miRs-15 and -133. Secondly, several miRNAs have been described to induce (miR-210) or repress (miR-15, -24, -26, and -17–92 cluster) angiogenesis. Furthermore, miRNAs modulate cardiac remodeling via inhibiting (miR-15 and -34) or enhancing (miR-1, -21, -24, -126, -155, -221, and -499) the function of stem and/or progenitor cells.

A recent manuscript by Shen and colleagues identifies a novel and crucial role for the miR-30 family in acute MI injury and cardiac function (8). In this study, the authors

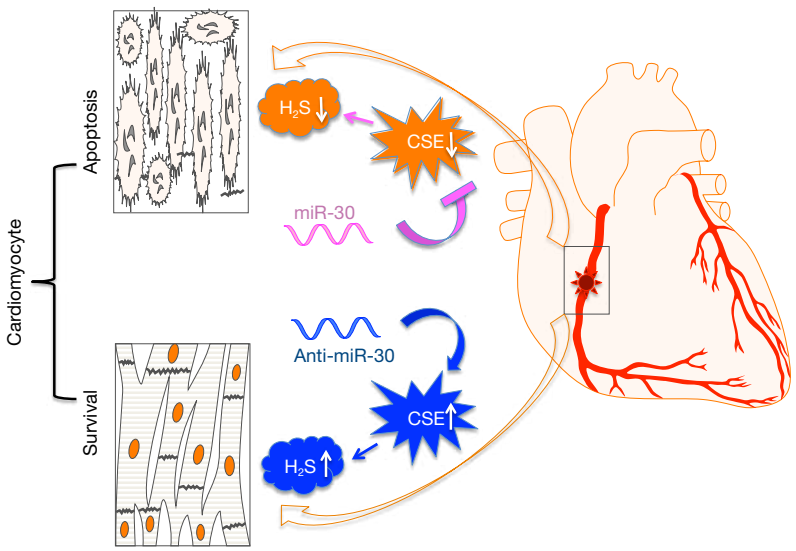

Figure 1 Therapeutic effect of anti-miR-30 on cardiomyocyte survival in myocardial ischemia.

emphasized on the cardio-protective role of hydrogen sulfide (H_2S), predominantly deriving from L-cysteine and being catalyzed by cystathionine-c-lyase (CSE). Their experiments provide additional evidence that the miR-30-CSE-H_2S axis contributes to the protection against cardiomyocyte ischemic injury, both *in vitro* and *in vivo*, by regulating H_2S production.

The authors were able to discover significant deduction of CSE and H_2S in MI, which negatively correlates with expression levels of the miR-30 family. Of importance, they could confirm that CSE is a direct target of the miR-30 family. The miR-30 family regulates CSE mRNA and protein levels, thus affects H_2S production and hypoxia-induced cardiomyocyte injury. In addition, silencing the miR-30 family not only protects against cardiomyocyte apoptosis during hypoxia, but also increases cardiac function evaluated by echocardiography after MI induction in mice, which turns out to be a CSE-dependent mechanism, as these effects were absent in genetically mutated CSE knockout mice. In contrast, delivery of miR-30b in mice greatly aggravated MI injury. Taken together, the study is able to demonstrate for the very first time that the miR-30 family regulates H_2S production by directly targeting CSE. Inhibition of the miR-30 family after MI injury offers clear therapeutic value to 'keep our heart going on' (*Figure 1*).

One limitation to the translational feasibility of the study is that the authors did not evaluate potential off-target effects in other organ systems than the targeted heart (e.g., liver, kidney) in which systemically administered miRNA modulators assimilate to a much higher extent. Further translationally focused pre-clinical studies should take these considerations into account. One promising solution may be local delivery of miRNAs highlighted in the past by Hinkel *et al.* (9). In their study, locked nucleic acid-modified antisense miR-92a (LNA-92a) was applied either regionally (antegrade or retrograde) with a catheter or systemically (intravenously). They confirmed that LNA-92a reduced miR-92a expression in the infarct zone regardless of the application venue. However, catheter-based delivery, but not intravenous infusion, reduced the infarct size compared with control LNA—treated pigs, which correlated with an improved ejection fraction and left ventricular end-diastolic pressure, while not accumulating in other organs (such as kidney and liver). In addition, as reported by previous studies (10,11), both down-regulated miR-30a and up-regulated miR-30c/d could aggravate myocardial hypertrophy, indicating that distinctive modulation of a miR-30 family subtype would need to be taken into account when developing future therapeutic cardiomyocyte rescue strategies after acute MI in humans. Last but not the least, endogenous H_2S level subsequent to miR-30 modulation should be tightly controlled due to its latent toxicity.

The therapeutic value of miRNA in cardiovascular as well as other diseases is obvious. By having unique expression profiles and higher stability in biological samples, miRNAs quickly emerged as novel biomarkers.

The ability to regulate multiple genes in various disease-contributing signaling pathways makes them promising future therapeutic targets. Noteworthy, translational applicability to utilization in humans needs to be viewed with caution, especially issues relating to potential immune stimulatory effects, mode of delivery, and off-target effects. These obstacles need to be overcome to bring miRNA therapeutics into mainstream clinical practice.

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Footnote

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MicroRNA-499-5p: a therapeutic target in the context of cardiovascular disease

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Cardiovascular disease remains a major cause of death worldwide. Atherosclerosis, narrowing of the arteries due to the accumulation of cholesterol in macrophages within the sub-endothelial space of the vessel wall, is the primary underlying cause of cardiovascular disease. Hypercholesterolemia is therefore an established risk factor for the development of atherosclerotic lesions and cardiovascular disease (1,2). Other major cardiovascular risk factors include smoking, high blood pressure, and the presence of type II diabetes (1,2). Unfortunately, it is still difficult to reliably predict the risk for the development of cardiovascular disease and the associated mortality in individual subjects. A clear need therefore exists for the discovery of sensitive cardiovascular disease biomarkers.

MicroRNAs constitute a large family of highly conserved non-coding RNAs that inhibit the protein expression of their target genes through modulation of the mRNA translation rate and/or stability (3). Initial findings from the oncology research field have indicated that these ~22 nucleotide long RNA species may be useful as biomarkers since several tumorous tissues display a specific change in their microRNA expression profile as compared to their related non-diseased tissues (4-7). In follow-up studies we have been able to show that the presence of cardiovascular disease in humans subjects, e.g. in unstable and stable angina pectoris patients, coincides with a change in the microRNA profile of peripheral blood mononuclear cells (PBMCs) (8). More specifically, we observed that the relative expression levels of miR-135a and miR-147 were respectively 5-fold higher and 4-fold lower in PBMC fractions from coronary artery disease patients as compared to those of unaffected controls. Our studies also revealed that the PMBC fractions of stable and unstable angina pectoris patients could be distinguished based upon the expression level of three specific microRNAs. Relative expression levels of miR-134, miR-370, and miR-198 were higher in unstable subjects that had experienced ischemic chest pain at rest within the preceding 48 h versus those that had stable effort angina of >6 months duration. It thus appears that microRNAs may be able to also serve as biomarkers of (unstable) coronary artery disease.

In the context of our previous observations, in this commentary, I would like to highlight the paper by O Sullivan *et al*. that has recently been published in the International Journal of Cardiology (9) as it provides strong novel support for the predictive power of microRNA signatures in the cardiovascular disease setting. Similar as in our study, O Sullivan *et al*. aimed to show the differential expression of microRNAs in patients with stable coronary artery disease, unstable coronary artery disease and control subjects with normal coronary angiograms. Their unstable group of patients consisted of subjects who presented with ST-Elevation Myocardial Infarction (STEMI) as diagnosed per 2013 ACCF/AHA Guidelines for the Management of

ST-Elevation Myocardial Infarction. All STEMI group inclusions therefore had presented within 12 h of chest pain onset, undergone primary percutaneous intervention (PCI), and had coronary artery occlusion confirmed by angiography. A clear improvement as compared to our studies was that O Sullivan *et al*. included such a high number of patients that they could also correct for possible interactions with known cardiovascular risk factors. Levels of miR-93-5p (increased), miR-146a-5p (decreased), miR-16-5p (increased), and miR-15a-5p (increased) were all significantly changed in plasma specimens of stable coronary artery disease patients as compared to those obtained from controls (9). However, the most striking finding of the study by O Sullivan *et al*. was that inclusion of plasma miR-499a-5p levels (increased in STEMI patients versus controls) in the prediction model significantly enhanced the sensitivity as compared to traditional risk factors alone to identify subjects suffering from myocardial infarction (9). As such, the data from O Sullivan *et al*. suggest that high plasma miR-499a-5p levels may serve as a complementary biomarker for the presence of acute coronary syndromes.

Expression profiling in pigs has suggested that miR-499-5p is highly conserved and preferentially expressed in the myocardium (10). MiR-499-5p is therefore generally regarded to be a cardio-specific microRNA in humans. Importantly, the observation by O Sullivan *et al*. that the presence of the cardiac microRNA miR-499a-5p in plasma contains high predictive power in the context of acute coronary syndromes does not stand by itself. Olivieri *et al*. showed that median circulating levels of miR-499-5p were significantly higher at admission in acute myocardial infarction patients that died within the following year as compared to those that survived the cardiovascular event (11). Furthermore, a step-wise increase in plasma miR-499-5p levels was observed as compared to healthy controls in subjects suffering from acute heart failure without evidence of acute myocardial infarction and those that did display acute non-ST elevation myocardial infarction (12,13). Moreover, studies by Gidlöf *et al*. (13) and D'Allesandra *et al*. (14) have indicated that miR-499-5p levels are transiently elevated in plasma of human subjects in response to the development of myocardial infarction. In further support of the notion that miR-499-5p levels can be used as a highly sensitive biomarker of acute cardiovascular events, Gidlöf *et al*. also observed that circulating miR-499-5p levels can reliably predict the presence of STEMI (13,14).

In agreement with the aforementioned human findings, induction of myocardial infarction induced a rapid, but transient, increase in plasma levels of miR-499-5p that peaked at 24 hours after the coronary artery occlusion in both mice and pigs (13,14). The miR-499-5p plasma profile in the murine myocardial infarction model was not mimicked by that of other microRNAs supposedly expressed specifically in cardiac muscle (14). The increase in plasma miR-499-5p levels is thus probably not due to a non-specific secretion of the microRNA from cardiac tissue, e.g. in response to myocardial infarction-associated necrosis of cardiomyocytes. Interestingly, a rapid decrease in miR-499-5p levels has been detected in response to hypoxia in cultured rat cardiomyocytes *in vitro* (15). Based upon these combined findings, one can assume that a decrease in cardiomyocyte miR-499-5p levels and concomitant rise in plasma miR-499-5p levels may therefore be a general biomarker of cardiac distress. A higher plasma level of miR-499-5p thus would associate with a higher degree of cardiac dysfunction. In accordance, athletes that have immensely challenged their heart through running a marathon also display a transient rise in circulating miR-499-5p levels (16).

The question remains as to whether the increase in plasma miR-499-5p levels is only a biomarker of cardiac dysfunction/hypoxia or if this microRNA actually plays a role in the pathogenesis of acute cardiovascular events. In their elegant study, Li *et al*. have recently addressed this issue. Overexpression of miR-499-5p in cultured cardiomyocytes lowers programmed cell death protein 4 (neoplastic transformation inhibitor; PDCD4) mRNA expression which translates into a decreased apoptosis rate, while miR-499-5p inhibition increases PCDC4 transcript and protein levels and induces cardiomyocyte apoptosis (15). In line with an inverse relation between miR-499-5p levels and cardiomyocyte death, a lower miR-499-5p expression can be found in infarcted (dying) versus non-infarcted (healthy) cardiac tissue (15). Importantly, overexpression of miR-499-5p in cardiomyocytes was able to protect the heart against myocardial infarction-associated tissue damage *in vivo*. A remarkable ~50% decrease in infarct size was noted in miR-499-5p agomir-treated mice as compared to controls (15). It has been suggested that microRNAs circulating in the plasma compartment, i.e., in membrane vesicles, can be transferred to recipient cells to facilitate cell-to-cell communication (17). When taking the *in vitro* and *in vivo* findings from Li *et al*. into account, it can be hypothesized that, under myocardial infarction conditions, hypoxic cardiomyocytes release miR-499-5p for subsequent transfer to and incorporation by unaffected cells to confer

protection against myocardial infarction-associated cellular apoptosis and tissue death.

In conclusion, the study by O Sullivan *et al*. has (1) provided substantial new support for the relevance of miR-499-5p as non-invasive biomarker of acute coronary events and (2) highlighted the general potential of circulating microRNAs as predictors of disease. The recent discovery that miR-499-5p may play a protective role in cardiomyocytes has opened up new possibilities to treat subjects at risk of developing acute cardiovascular syndromes. Thus far, phase I and II clinical trials involving microRNA-based therapies, i.e. treatment of cancer patients with a liposome-formulated mimic of the tumor suppressor miR-34 (MRX34), have not yet yielded valuable drugs (18). However, it is conceivable that therapeutic approaches aimed at increasing plasma miR-499-5p levels will be developed in the future that can be of benefit for high risk cardiovascular disease patients.

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Early detection of myocardial infarction—microRNAs right at the time?

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Acute myocardial infarction (AMI) is the major cause of death and disability worldwide and the risk of death is highest within the first hours from chest pain onset (1). Hence, an early and accurate diagnosis of AMI in patients presenting with chest pain is of paramount importance for effective treatment and improvement of outcome prognosis (2). In addition to the clinical symptomatic, electrocardiographic and imaging findings, the rise or fall of cardiac troponins, released from the damaged cardiomyocytes, are important biomarkers for the diagnosis of AMI (2). However, troponins can also be released in other cardiac pathologies, such as myocarditis, and chest pain is often misunderstood or difficult to allocate by the patient. Therefore, novel biomarkers are needed to fine-tune AMI diagnosis and potentially enable personalized treatment in the future.

Implementation of novel biomarkers into clinical practice is an important area of biomedical research. A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention, and the overall expectation of a biomarker is to enhance the ability of the clinician to optimally manage the patient (3). To evaluate a biomarker, important measures are its sensitivity (the ability to detect the disease, i.e., identification of true-positives) and specificity (the ability to the detect absence of disease, i.e., identification of true-negatives), as well as its positive and negative predictive values (3).

Recently, new technologies have been applied for screening potential novel biomarkers, taking into account not only proteins but also additional promising molecules such as nucleic acids (4). From these analyses, non-coding RNA species have been characterized including microRNAs (miRNAs).

miRNAs are small sequences of endogenous RNA molecules regulating gene expression at a post-transcriptional level (5). miRNAs are involved in a broad range of biological processes and their dysregulation impacts disease development (5). Because of their remarkable stability in biological fluids such as serum/plasma and urine (5) and their tissue-specificity, there is a great interest in miRNAs as novel circulating biomarkers.

Several lines of evidence support this role of miRNAs: studies—using human blood samples as well as animal models, and focusing on the diagnostic potential, compared miRNAs in AMI patients to controls as well as to established biomarkers e.g. troponin (6-9). The most-reported miRNAs were the cardiac-specific miRNAs miR-1, miR-133a, miR-208b and miR-499 (10) that showed high sensitivity and specificity as biomarkers for AMI (10). Similar, miRNAs were identified that facilitate differentiation of unstable angina pectoris (UAP) from stable AP (SAP) or non-coronary chest pain (NCCP). These include the non-cardiac specific miRs 134, 198, 370 (UAP/SAP) and miRs

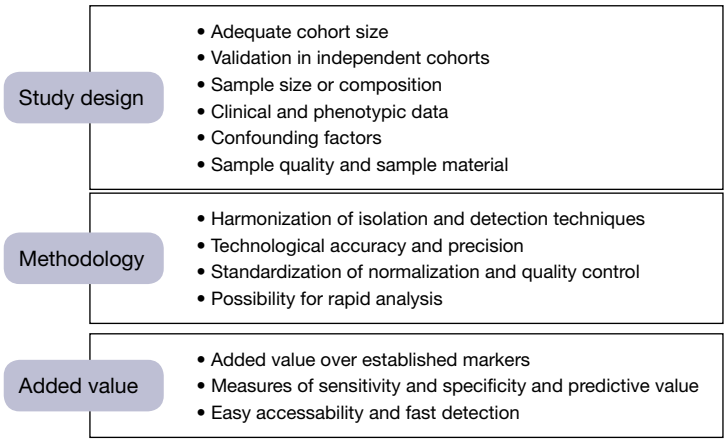

Figure 1 Main challenges to overcome in miRNA analyses for diagnostic purposes. miRNA, microRNA.

132, 150, 186 (UAP/NCCP) (11), indicating that also non-cardiac specific miRNAs—in combination - can be of diagnostic value.

In this issue of Cellular Physiology and Biochemistry Wang *et al*. (12) investigated a signature consisting of three (non-cardiac) circulating miRNAs, namely miR-19b-3p, miR-134-5p and miR-186-5p, for its potential use as a biomarker for (fine tuning) AMI diagnosis. The authors present results of a longitudinal assessment of these three miRNAs over different time points after chest pain onset and explored the diagnostic potential of the single miRNAs and in combination. All three miRNAs were upregulated after the onset of chest pain within a range of four to 72 hours, and showed a positive correlation with cardiac troponin I (cTnI). Not surprisingly, levels of cTnI were also increased already at the time of admission. Calculation of the area under the ROC curve (AUC)—as an indicator for the discriminatory performance of the miRNAs, showed good AUC values indicating a discriminatory ability of the three miRNAs—singly and as a combined miRNA score. Interestingly, Wang *et al*. also investigated the influence of heparin and medication on the circulating levels of the three miRNAs and concluded that neither administration of heparin nor the administration of ACE inhibitors, beta-blockers, mitrates, statins, aspirin and clopidogrel) had an influence on the miRNA levels.

Although the concept to explore miRNAs for diagnosis of AMI is very attractive, there are still various challenges to face due to preanalytical and analytical factors influencing data quality. These factors include the study design, the choice of material, isolation, detection and processing techniques as well as normalization strategies, and the influence of drugs and other, non-cardiac disease and phenotypes (*Figure 1*).

An appropriate study design is a foremost requirement for reliable biomarker identification, ensuring adequate sample size for analysis and accounting for possible confounders (5). Wang *et al*. (12) used a comparatively small group of AMI patients and controls. Aside from the small study cohort, other studies of that purpose usually match one or even two control subjects to one "case" according to a panel of pre-defined factors, rather than merely excluding differences of the overall groups or validate their initial findings in much larger independent study cohorts. The three miRNAs investigated in the study of Wang *et al*. (12) have all been described already to be involved in development as well as pathologies of the heart (13), such as heart failure, but also in other context, such as gastric cancer (14). Therefore, great attention needs to be paid to the selection of case as well as control groups and potential confounding factors influencing miRNA levels. Furthermore, only a small number of non-cardiac miRNAs have been selected by the authors, apparently based on the current literature. Thus, other miRNA with potentially better performance might have been missed.

The detection of miRNAs currently relies on real-time PCR techniques, and thus depends on the limitations raised by the current lack of standardized isolation, detection and normalization strategies. To date, several approaches are used including manual or commercial isolation techniques, different reverse transcription and PCR approaches as well as different normalization methods such as the use of synthetic spike-in material (as in the current study) or the use of an average of the Ct values of all tested miRs (4,5).

To eliminate this technical and analytical variability and thereby avoiding artifactual data generation, consensus on standard methods for all steps is imperative.

Of great interested is that Wang *et al*. (12) directly investigated the effect of heparin and medication on the miRNA levels of interest. However—surprisingly—no significant influence on miRNA levels were found, although at least for miR-19b-3p and miR-186-5p a trend was observed which might reach statistical significance with a higher number of patients tested. Indeed, several other studies have demonstrated effects of statins and anti-platelet drugs on the levels of various circulating miRNAs (15). And heparin is known to interfere with a number of diagnostic markers and had previously been described to alter circulating miRNA levels (16,17).

For the translation of a potential biomarker candidate into clinical application it is of importance to investigate whether the novel marker shows an added value to established markers. For this, potential novel biomarkers for the diagnosis of AMI, i.e., circulating miRNAs, need to be compared directly to cardiac troponins—the gold-standard biomarker currently used for diagnosis of AMI. Since the introduction of highly sensitive troponin assays into clinical practice already substantially improved the early diagnosis of AMI, a direct comparison of the circulating miRNAs to high sensitivity assayed troponins is mandatory. Also, to accurately judge the diagnostic performance of circulating miRNAs, measures such as sensitivity, specificity as well as negative and positive predictive values are needed.

Lastly, independent of the purely methodological aspects, research is also required to address other relevant questions concerning the translation of miRNAs into clinical practice, such as a feasible time frame for qPCR analysis before patient treatment, ethical issues as well as optimal cost-benefit analysis.

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Footnote

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Circulating micro ribonucleic acids in cardiovascular disease: a look beyond myocardial injury

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Laboratory markers are of significant clinical importance in the evaluation of patients with suspected cardiac diseases. They have evolved as essential tools in cardiology over the last 50 years, i.e., lipid testing for primary and secondary prevention, creatine kinase isoenzyme MB and subsequently the more sensitive and specific cardiac troponin (cTn) testing for the diagnosis and management of acute myocardial infarction (AMI), and more recently natriuretic peptide (NP) testing for the diagnosis (in particular exclusion), risk stratification, and monitoring of heart failure (HF) (1-3). We are beginning an era when it may be possible for biomarkers to direct treatment to optimize patient management. This is already the case with cTn (1,4) but should be the final goal with all cardiac biomarkers. However, there are still some open major clinical issues, e.g., the diagnosis of myocardial ischemia. Despite huge research efforts in recent years, which were triggered by the great clinical significance and economic impact of cardiac diseases, biomarkers for the prediction of coronary artery disease (CAD) and for risk stratification in stable CAD or the general population have not yet fulfilled their manifest promise so far (5). The most established marker in this respect is high-sensitivity C-reactive protein (hs-CRP) which still remains controversial (3,6).

The 1990s were the golden era of cardiac biomarkers with the implementation of cTn and NP routine testing. Numerous additional biomarkers were discovered and immunoassays were developed which were also suitable for routine measurement. The main focus was on markers of coronary plaque formation, plaque destabilization (e.g., myeloperoxidase), intracoronary thrombus formation (coagulation and platelet activation, reduced endogenous fibrinolytic activity), and markers of myocardial ischemia (e.g., ischemia modified albumin). However, the vast majority of these markers did not make the way from research to routine application due to either preanalytical, analytical issues, or because the clinical impact for risk stratification was limited as these markers did not add much to traditional risk factors and even in multimarker approach improved risk stratification and patient reclassification only very modestly above established routine biomarkers (5). Importantly, they did not lead to direct information about how to improve patient management. More recently, copeptin, a very unspecific marker of endogenous stress, was suggested for rapidly ruling out AMI in the emergency department. However, no significant benefit compared to high-sensitivity cTn (hs-cTn) testing could be convincingly demonstrated (7). During this period also genomic biomarkers entered the field and have been particularly popular in the last two decades. Almost all of the candidate-gene era genetic biomarkers of cardiovascular disease failed to be validated after an initial period of enthusiasm (8). Rare variants may be potent but because they are rare, they do not identify large numbers of additional patients at risk. Common variants such as single genetic variants confer extremely small risks such that the usual way of calculating

risk by assessment of traditional cardiovascular risk factors is better than analyses for these commonly occurring variations in deoxy ribonucleic acid (DNA) sequences. Consequently, the current consensus is not to test for commonly occurring genetic variants with weak effects (9).

Another currently very popular research topic is circulating plasma micro ribonucleic acid (miRNA) testing (10). miRNA are small (typically less than 25 nucleotides), single-stranded, endogenous, non-coding RNAs that post-transcriptionally regulate gene expression by destabilizing messenger RNA (mRNA) or translation repression and thereby preventing proteins synthesis (11,12). Interestingly, each miRNA can target several mRNA while each mRNA can be targeted by multiple miRNAs (12). Eventually miRNAs are secreted from cells into blood being packaged in microparticles, but they are also found bound with proteins or high-density lipoproteins. The biological function of circulating miRNAs remains to be established. It is unclear whether circulating miRNAs are messengers in the cell-to-cell communication with active secretion or merely degradation products without any biological function with passive release as necrosis associated biomarkers.

More than 1,000 miRNAs have been identified in the human genome, but based on their tissue distribution and physiological function in the regulation of angiogenesis, apoptosis, and cell differentiation and proliferation miRNA-1, -133, -145, -208, and -499 appear to be most promising candidate markers for testing their diagnostic and prognostic potential in cardiovascular diseases (10). Regarding cardiac-specificity miRNAs-208 and -499 are promising, and in fact, particularly miRNA-499 and miRNA-208b were evaluated in patients with suspected acute coronary syndromes (ACS) with a rapid increase early after AMI with a high sensitivity within 3 hours from symptom onset (13). The hope still is to identify a miRNA profile (e.g., miRNA-1, -499, and -21) specific for myocardial ischemia (14), which would be of particular clinical interest. First studies, however, could not demonstrate an additive value of miRNA to hs-cTn testing for AMI diagnosis (13). In patients with CAD miRNA-132, miRNA-150, and miRNA-186 appear to be associated with ACS (15), and miRNAs (e.g., miRNA-145) appear to be associated with presence of CAD as well (16). However, the published data on the value of miRNAs for diagnosis and in particular for risk stratification in various cardiac diseases is still contradictory and inconclusive (10), and large clinical studies with appropriate pre-analytics and analytics remain to be done to demonstrate the additive value of miRNA measurement to conventional cardiac biomarker testing convincingly.

Currently miRNA testing is also time consuming with demanding pre-analytics and analytics (10), which precludes widespread routine use. It is very important to prepare cell free plasma to avoid in-vitro contamination from blood cells, but the methods of plasma preparation are frequently not sufficiently given in publications. Hemolysis must be avoided during blood collection and should be ruled out by oxyhemoglobin testing before miRNA testing in plasma samples. Whole blood must be processed immediately for plasma preparation as well. *In vitro* miRNA contamination from blood cells may be a particular problem if miRNAs are tested in stored frozen plasma samples which were not collected and prepared with the aim of testing miRNAs, and consequently this may lead to erroneous results and publications. Heparin plasma is not suitable for miRNA testing because heparin may inhibit complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR), and quantitative reverse transcription PCR (qRT-PCR) is the most widely used method for circulating miRNA determination. Thus, it is also important to know whether patients were treated with heparin before blood collection. Another unresolved issue is the lack of harmonization of methods and of test result normalization (e.g., synthetic spike-in control miRNAs *vs.* expression or mean expression value of one or better a panel of commonly expressed miRNAs in a sample that are not associated with diseases), which makes it very difficult to compare published study results. Synthetic spike-in RNAs have the additional advantage that this can be also used to monitor the efficiency of RNA isolation, cDNA synthesis, and PCR amplification as well as to reveal potential presence of nucleases in the sample.

In conclusion, the role of biomarkers in cardiovascular diseases, such as AMI and HF, is very well established with cTn and NP testing as essential parts of patient evaluation with suspected AMI or HF (1,2,17,18). Given this powerful role of established cardiac biomarkers it is very difficult to demonstrate a significant benefit of add-on testing of new biomarkers compared with established markers (i.e., hs-cTn, NP, and hs-CRP) in cardiac diseases. Therefore, as with other heavily investigated novel markers, the coming years will show whether miRNA testing will make the way from research to routine use after an initial hype at the beginning of research, particularly as hs-cTn assays already entered routine use (17) and as even more sensitive research cTn assays ("ultra-sensitive") have been developed with

significant clinical potential (17,19). In contrast to standard cardiac biomarker testing including ultra-sensitive cTn a lot of pre-analytical and analytical issues of miRNA testing also have to be solved before routine testing is feasible.

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Clinical utility of novel biomarkers in acute myocardial infarction

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Over the past decades, biomarkers of myocardial injury, particularly cardiac troponin (cTn), creatine kinase and its isoenzyme creatine kinase myocardial band, have been extremely valuable for the diagnosis and clinical decision making in patients with suspected acute coronary syndrome (ACS). These established markers of necrosis were recently complemented by numerous novel biomarkers reflecting causes and consequences of myocardial infarction (e.g., inflammation, endothelial dysfunction, or hemodynamic stress). Therefore, research efforts have been directed to determine the additional diagnostic and prognostic value of these novel biomarkers.

While the diagnosis and management of ST-elevation myocardial infarction is solely based on clinical and electrocardiographic findings, the identification of patients with non-ST-elevation myocardial infarction (NSTEMI) requires a more sophisticated approach including the measurement of cTn and other biomarkers of myocardial necrosis (1,2). In patients with NSTEMI, levels of cTn usually rise rapidly within 1 hour after symptom onset and remain elevated for a variable period of time (1). Advances in technology have led to an improvement in cTn assays and have refined the ability to detect and quantify myocardial injury. By using conventional, non-high-sensitive cTn (hs-cTn) assays elevated troponin concentrations in the peripheral blood may be detected delayed which necessitates serial testing to ascertain the diagnosis in most cases. Novel biomarkers were thought to bridge this "troponin-blind" gap and enable immediate decision making in patients presenting with acute chest pain.

Copeptin has emerged as one of these promising biomarkers that may overcome this lack of sensitivity within the first hours after symptom onset although non-specific to myocardial injury. It is released in response to endogenous and/or hemodynamic stress and a dual-marker strategy with conventional cTn and copeptin showed a high negative predictive value for the early rule-out of NSTEMI including a reduction of the average time-to-diagnosis, therefore adding incremental value to conventional cTn assays (3,4). However, the introduction of hs-cTn assays in clinical routine significantly increased the diagnostic performance of cTn and facilitates to rule out myocardial infarction within 1 hour (1). An additional diagnostic value of copeptin to hs-cTn has not been convincingly established (5,6). Consequently, current guidelines recommend the routine use of copeptin for the early rule-out of NSTEMI only if hs-cTn assays are not available (1).

In view of the limited diagnostic gain in addition to hs-cTn, the focus shifted to the prognostic value of novel cardiac biomarkers. In the British Medical Bulletin, Feistritzer and colleagues provide a systematic review of selected biomarkers reflecting myocardial injury, inflammation/fibrosis, and hemodynamics (7). The authors illustrate the pathophysiological background and the prognostic utility of hs-cTn, natriuretic peptides, copeptin, galectin-3, corin, fetuin-A, adiponectin and micro-RNAs. Scientific advances created numerous other markers that may reflect further aspects of coronary artery disease (e.g., plaque

destabilization/rupture or platelet activation) but would exceed the scope of this review (8). In the context of prognostic utility, growth differentiation factor 15 (GDF-15) showed promising results as an independent predictor of mortality and a potential tool to guide therapy and should therefore be mentioned additionally (8,9). However, although most of these novel biomarkers can improve risk stratification in patients with acute myocardial infarction to some extent, the question regarding their actual role in clinical routine arises (8). As nicely illustrated by Feistritzer and colleagues, the obligatory measurement of hs-cTn already provides substantial prognostic information and is pivotal for patient management (7). In addition, only natriuretic peptides were extensively validated and have proven prognostic utility on top of cTn although without definitive treatment implications (10). Other novel biomarkers have not yet shown useful regarding therapeutic decision making and their incremental prognostic value over and above established biomarkers and risk scores is only marginal. Similarly, multi-marker approaches involving several novel biomarkers failed to clearly outperform the prognostic usefulness of cTn (11). Therefore, guidelines for the management of patients with acute myocardial infarction do not recommend their routine assessment (1,2).

Several aspects might explain why novel biomarkers have not yet found their way into clinical routine. Established biomarkers, basically hs-cTn and natriuretic peptides, as well as clinical scores such as the Global Registry of Acute Coronary Events (GRACE) or the Thrombolysis In Myocardial Infarction (TIMI) Risk Score enable excellent risk stratification, are widely available and cost effective (1). Moreover, imaging modalities provide further prognostic insights. Echocardiography to evaluate left ventricular function and contraction abnormalities is recommended in all patients with acute myocardial infarction (1,2). Cardiovascular magnetic resonance (CMR) imaging allows for a more detailed tissue characterization and is increasingly available in clinical routine. Parameters derived from these imaging modalities, particularly CMR, have demonstrated incremental prognostic information regarding hard clinical endpoints whereas many biomarkers have only been linked to impaired left ventricular function or adverse remodeling (12). Therefore, the combination of established biomarkers, clinical risk scores and imaging parameters facilitates a sufficient risk stratification from a clinician's point of view. Novel biomarkers have to outperform these markers regarding sensitivity, specificity and/or a quicker release kinetic while being similarly cost effective and easily available to play a role in clinical practice. Since this is very difficult to achieve a potential alternative application of novel biomarkers is the guidance of treatment decisions to enable a more tailored patient management. However, a benefit of biomarker based approaches remains to be proven.

In summary, several novel biomarkers reflecting different pathophysiological aspects of acute myocardial infarction emerged during the last decade. However, few of them have proven valuable in clinical routine given the excellent diagnostic and prognostic performance of established evaluation strategies including cTn, clinical risk scores and imaging markers of myocardial damage. While cTn is highly specific for myocardial necrosis, it provides no information on the etiology of myocyte death. Thus, more research is necessary to determine new thresholds or additional helpful biomarkers to distinguish between etiologies in the heterogeneous field of ACS (e.g., in patients with atrial fibrillation, hypertension, Takotsubo syndrome, and/or kidney disease). Finally, an individualized biomarker-guided management according to cTn and other promising biomarkers like copeptin and/or GDF-15 (e.g., immediate coronary intervention in NSTEMI patients with high-risk biomarker features) should be addressed in upcoming ACS trials to evaluate if there is room for other novel biomarkers for clinical decision making next to cTn.

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Footnote

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Circulating microRNA biomarkers for cardiovascular risk prediction: are we approaching clinical application?

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Patients at high risk of cardiovascular morbidity and mortality are still difficult to identify. Unfortunately, the most widely used standard risk prediction models predict cardiovascular disease (CVD) rather poorly, since these risk calculators are mostly driven by age. Therefore, most young subjects are regarded as low risk merely based on their age, despite sometimes quite obvious adverse risk factors. Circulating biomarkers that can help to improve the identification of individuals at risk are therefore highly needed.

MicroRNAs are short noncoding RNAs that post-transcriptionally regulate gene expression (1). When microRNAs are shed into the circulation, they remain there in a stable state and reflect ongoing processes at a cellular level in the tissues (2,3). Because of these properties, circulating microRNAs have become increasingly popular over the years.

After many small, exploratory studies with divergent results, presently, also larger studies on circulating microRNAs are being published. In a recent study, Karakas *et al*. (4), assessed the prognostic value of 8 circulating microRNAs that had previously been identified to facilitate the diagnosis of unstable angina pectoris (UAP) (5) in a cohort of 430 acute coronary syndrome (ACS) patients and 682 stable angina pectoris (SAP) patients. The authors concluded that 7 of these 8 measured microRNAs are strongly predictive of cardiovascular mortality with areas under the receiver-operating curves (AUCs) up to 0.76; this represents an important step forward in the circulating microRNA field. However, these studies still struggle with many common pitfalls in microRNA measurement and interpretation of the results remains challenging.

Pitfalls in the measurement of circulating microRNAs and poor reporting on reverse transcription quantitative polymerase chain reaction (RT-qPCR) methods

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is the most common way to reliably assess microRNA levels. However, the way in which RT-qPCR measurements are performed and analyzed can have a large influence on the results. It is therefore of great importance to have standardized and validated methods for RT-qPCR microRNA measurement. The authors of the study of Karakas *et al*. (4) thoroughly report on statistical methods, but do not report on the details of the RT-qPCR measurement nor the handling of missing data. It is important to describe the RT-qPCR methods following the MIQE guidelines (6) and to describe how missing data was handled. Differences herein can lead to serious flaws and biases in the results, as explained more in detail below.

Handling missing data and low values

MicroRNAs often circulate in a very low concentration.

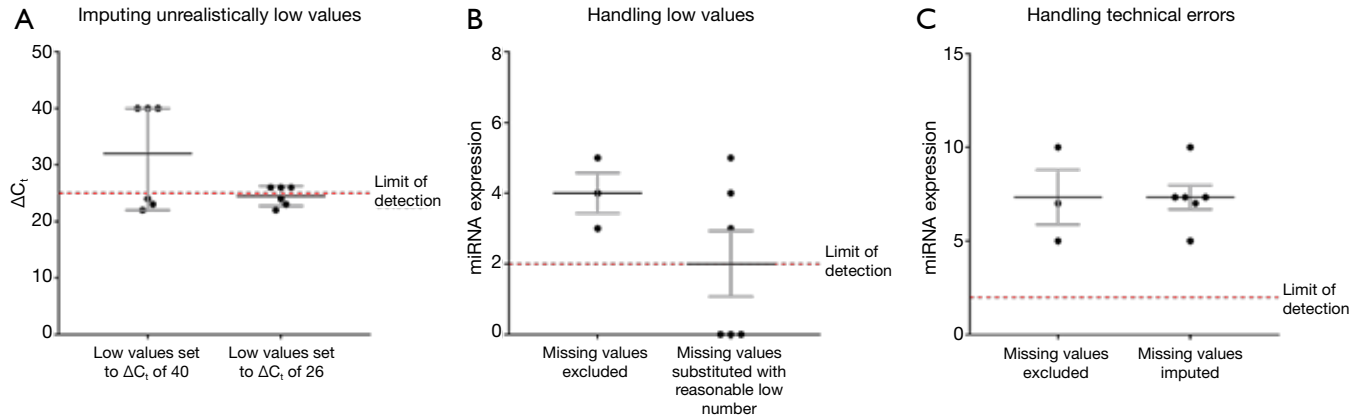

Figure 1 Pitfalls of microRNA data handling. The following pitfalls can be expected from wrong data handling. (A) shows the effect of using an unrealistically low value to substitute C_t values ≥40. Due to a large gap between the lowest measurable ΔC_t of 24.99 and the substituted ΔC_t of 40, there is more variation in the data, creating bias in the analysis. Dots are fictional data with mean (black line) and standard deviation (grey whiskers). This is solved by using a more appropriate ΔC_t to substitute these low values such as a ΔC_t of 26; (B) shows the effect of excluding missing values due to low microRNA expression. When these values are excluded, this falsely leads to increased expression levels. Therefore, missing values due to low microRNA expression must be substituted with a low value (e.g., 0); (C) shows the effect of excluding missing values that occurred due to technical errors. Excluding instead of imputing these missing values from the analysis will lead to an increased standard error of the mean and may therefore mask significance. In (B) and (C), dots are fictional data with mean (black line) and standard error of the mean (grey whiskers).

Even highly sensitive methods like RT-qPCR, need to be stretched to their limit of detection and sometimes certain microRNAs are even totally absent from the circulation. This most often results in missing values that complicate the measurement of microRNAs, since microRNAs can also appear to be missing due to a technical error.

It is most important to distinguish missing values due to a low concentration or complete absence of the microRNA from the circulation, from values that are missing due to a technical error. Missing data due to a low concentration is not missing at random and therefore should be substituted with a low value, whereas missing data due to technical errors occur at random and must be imputed.

The authors of Karakas *et al.* (4) acknowledge the issues related to a low microRNA concentration, since they substitute C_t values ≥40 with a ΔC_t of 40. Unfortunately, by doing so, they substitute the low microRNA concentrations with an unrealistically low value. We will explain this in detail. In the study, the formula C_t (microRNA) – C_t (cel-miR-39) was used to calculate the ΔC_t. To produce a reliable qPCR curve based on enough data points to calculate a C_t from, a minimum C_t value of 15 is needed. Therefore, the maximum ΔC_t that can be calculated is [(maximum C_t of the microRNA =39.99) – (minimum C_t cel-miR-39 =15)] =24.99. However, in case of a C_t value of ≥40, the authors set the ΔC_t to 40, creating a large gap between the lowest value measurable (ΔC_t of 24.99) and the substituted low value. This value will contribute tremendously to the mean expression level compared to more realistic substitution values such as a ΔC_t of 26 (*Figure 1A*). If not accounted for, these unrealistic values can influence the results of cox or logistic regression analyses.

Concerning missing values, the authors do not report whether they encountered this and how this was handled. A missing value can occur because the C_t is 0, meaning that the microRNA was under the detection limit, which also represents a low concentration. Therefore, C_t values of 0 should also be imputed with a reasonably low number, instead of simply excluding them, which could lead to false higher expression levels (*Figure 1B*).

A missing value can also occur due to a technical error. By excluding these data, the study will lose power and meaningful differences can be lost as illustrated in *Figure 1C*. The best way to handle this type of missing data is by a multiple imputation method, in which a weighted average is imputed, taking into account the characteristics of the study population. Failure to handle this issue, might lead to either under- or overestimation of the effect.

Single versus duplicate or triplicate RT-qPCR measurements

Another major issue concerning the reliability of RT-qPCR measurements of microRNA data is that often single measurements per sample are performed, where duplicate or even triplicate measurements would be more appropriate. Using measurements in duplicate or triplicate highly increases the precision of the microRNA measurements. Besides, replicate measurements are essential to distinguish missing data due to the technical errors from missing data because of low expression, as explained above.

If a single microRNA measurement shows a C_t value of 0, one would falsely decide that the microRNA is not detectable within the sample, whereas if the measurement would have been done in triplicates, the other two C_t values might have had values of for instance 32. In this particular example one would conclude, that the first measurement has a technical error and that the true value is the average of the other two measurements. On the other hand, when all three triplicates have a C_t of 0, it is most likely that this microRNA cannot be measured due to low expression. Unfortunately, the authors do not report whether their RT-qPCR measurements were single or multiple measurements.

Normalization methods

As the authors state in the discussion, another potential source of bias is the normalization with cel-miR-39. Since cel-miR-39 is not incorporated in microvesicles or protein or lipid complexes, variations in extraction of microRNAs from these vesicles and complexes are not accounted for (7). Therefore, we recently proposed a normalization methods using a panel of endogenous microRNAs, best representing stability of the data and taking in to account technical failures during the RT-qPCR measurement (8). We therefore suggest using these specific panels for specific samples.

Interpretation of the study results

The authors of the paper by Karakas *et al*. (4) nicely show that circulating microRNAs predict cardiovascular death in coronary artery disease. This is one of the first papers addressing such an important issue in such a large population. Besides, the observed association of 7 of the 8 microRNAs related to cardiovascular mortality, is fairly strong. On the other hand, because the authors do not report on the number of death and the data handling as discussed above, the observed results might be slightly overestimated. Remarkably, this important observation is mainly present in the ACS group, since when analyzing the overall group, the observed association is slightly attenuated.

The authors speculate on the pathophysiological process behind these microRNAs and state that they could be hypoxia markers. On the other hand, it appears from Table 2 in the paper, that the expression level of all the eight microRNAs in ACS are actually lower than in SAP (higher ΔC_t = lower expression level). Therefore, it seems that this intriguing suggestion cannot directly be support by these data. The authors show another intriguing observation, namely, that the association is influenced by vessel disease and left ventricular ejection fraction (LVEF). When the authors correct their data for the number of diseased vessels and LVEF, the association becomes stronger, meaning that this association is mainly present in individuals with a lower atherosclerotic burden and/or a preserved ejection fraction. This suggests that these microRNAs might reflect another underlying pathophysiological mechanism strongly related to cardiovascular death. Taken together, Karakas *et al*. importantly advance this field by showing predictive power well beyond currently used risk scores, and suggest that the circulating microRNAs measured are related to cardiovascular death. Obviously, more detailed analysis of the technical details of their RT-qPCR measurement may improve signal to noise ratio and further facilitate the use of these biomarkers.

Relation to other microRNAs studies

In this study, microRNAs that were previously associated with UAP were identified (5). Although only small studies have compared UAP with controls (9-11), none of these markers that were identified in the study of Zeller *et al*. (5), have been found previously. Additionally, since UAP is atherosclerosis without substantial ischemia, some overlap with previously found markers for SAP in earlier studies would have been expected. However, none of the eight microRNAs that were measured in the study of Karakas *et al*. (4) were found in a total of 17 earlier studies on SAP markers (12). Although, the study of Zeller *et al*. (5) is the first to consistently show an association of microRNAs with UAP in three independent cohorts, we maintain to wonder why so many different study of microRNA biomarkers

produce different results. This might be a cause of the many pitfalls of microRNA measurement, the lack of standard methods for measurements and normalization or handling of missing data. On the other hand the study of Karakas *et al*. (4) remains one of the largest and most thoroughly performed studies in the field of circulating microRNAs. Moreover, they found highly interesting markers for coronary artery disease risk prediction, despite the raised issues concerning data handling. However, subsequent external validation with more accurate measurements are needed to assess if these circulating microRNA biomarkers could be of value in clinical practice.

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Footnote

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Future Prospect

Exosomes: scytales in the damaged heart

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Exosomes and intracellular communication

As cells in our organism are constantly sending out and receiving signals, cell-cell communication is an essential way to maintain process homeostasis while allowing adaptation to external stimuli. Disturbances in cell-to-cell communication will result in disease. Cells communicate with each other via extracellular molecules such as nucleotides, short peptides, proteins or lipids that are released to the extracellular space and bind receptors on other cells, therefore inducing signaling and modifying the molecular status of the recipient cells. In addition to such molecules, cells also release membrane vesicles, representing a rich source of small molecules such as messenger RNAs, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), small amounts of DNA and low molecular weight lipids and proteins (including transcription factors and cytokines), all of which can also alter recipient cells that encounter such structures. Although initially thought as cellular debris and a sign of cellular death, in recent years more interest has been dedicated to extracellular vesicles (EVs) as mediators of long-range cellular communication by their presence in most body fluids. According to their size, EVs can be classified in microvesicles (MVs) (0.1 to 1 μm), exosomes (20 to 100 nm) and apoptotic bodies (ABs) (0.5 to 2 μm). (1) While MVs (and ABs) are assembled by budding from the plasma membranes, exosomes are raised from endosomal vesicles and formed as intraluminal vesicles by inward budding of the limiting membrane of the multivesicular bodies, which allows the internalization of small proteins, mRNAs, miRNAs and DNA. Once the multivesicular bodies fuse with the cell membrane, exosomes are released (2) and free to interact with target cells in different ways: (I) exosomes can fuse in a non-selective way releasing their content into the target cell; (II) hemifusion followed by a complete fusion between the exosome and the cell membrane; (III) internalization by phagocytosis; and (IV) specific interactions among exosomes and target cells mediated by extracellular matrix components (3).

It is important to underline that exosomes, although generated by different cell types expressing distinct markers, also carry common surface markers such as heat shock protein HSP70 and tetraspanins CD9 and CD63, making their characterization a difficult process (4). For research purposes, it is fundamental to purify EV populations, but despite a large body of literature describing protocols on this matter, a gold standard method for the isolation of exosomes remains to be established. This is a critical point while studying exosome-mediated cell communication since different isolation methods still result in different yield and purity populations (5).

Exosomes and intracardiac cell communication

Heart failure is one of the leading causes of morbidity and mortality worldwide (6). Cardiac remodeling is often a maladaptive mechanism that, independently of the nature of the initial insult, will ultimately cause ventricular dysfunction (7). Proper cardiac function does not only rely on cardiac muscle cells which account for one third of the total cell number of the myocardium, but rather on a

balance with other cell types including smooth muscle cells, endothelial cells, fibroblasts and immune cells. These very distinct cell types do not function isolated from each other but rather interact physically and/or via different autocrine, paracrine and endocrine factors. Indeed, the myocardium secretes exosomes, which are involved in intracellular communication in the adult heart (8). As extracellular spaces have mixed exosome populations from all kinds of cellular sources it is difficult to distinguish exosomes derived from a specific cell type from the intact organ. For this reason, *in vitro* studies have been very helpful not only providing evidence of exosome secretion but also in identifying the exosomal content and function from specific cell types. Early reports using primary cardiomyocytes and relevant cell lines have provided evidence of exosome secretion and detected nucleic acid–containing microvesicles/exosomes in the cell media, which could reprogram fibroblast gene expression (9,10). These investigations introduced a new concept in cardiac cell-cell communication, proposing that exosomes generated by cardiomyocytes are able to transfer protein or genetic information to neighboring cells of the heart.

Recent studies indicate that exosomal content is highly regulated by stress and disease conditions and despite the fact that research on cardiac exosomes is just emerging, a limited number of publications provide strong evidence that exosomes can exert pathological effects during cardiac response to stress as shown for different myocardial diseases as cardiac hypertrophy and peripartum or diabetic cardiomyopathy. Exosomes secreted from fibroblasts are enriched in miR-21* which once uptaken by cardiomyocytes will down-regulate Sorbin and SH3 domain 2 (SORBS2) or PDZ and LIM domain 5 (PDLIM5), both regulators of cardiac muscle structure and function, and induce cardiac hypertrophy (11). The fact that fibroblast-derived miR-21* induces cardiomyocyte hypertrophy also demonstrates that miRNA passenger strands can function as mature miRNAs (11). Another study demonstrates how, in women affected with peripartum cardiomyopathy (PPCM), a 16-kDa prolactin fragment (16K PRL) leads to the release of miR-146-enriched exosomes by endothelial cells (12). These exosomes can be uptaken by cardiomyocytes where miR-146a interferes with the physiological metabolism and contractile capacity of the cell, leading to the development of hypertrophy. Furthermore, levels of exosomal miR-146a were higher in plasma from patients with acute PPCM than healthy postpartum controls and patients with dilated cardiomyopathy. Maybe more important to underline is that standard heart failure therapy in PPCM patients lowered circulating exosomal miR-146a to control levels, indicating miR-146a as a strong potential biomarker for diagnosis and risk stratification of patients with PPCM (12).

As miRNA composition may reflect the metabolic or differentiation state of the exosome-producing cells, circulating exosomes found in body fluids such as plasma and serum are becoming an attractive tool for analytical studies and subsequent disease diagnosis (13). In agreement, the recent work of Chistiakov *et al.* (14), very elegantly underlines that differential exosomal content can lead to new cardiac-specific diagnostic markers by emphasizing the central role of exosomes in cardiac regeneration of the infarcted heart.

Exosomes and cardiac repair

The type of stress to which myocardial tissue is exposed seems to determine the content of the secreted EVs. In myocardial infarction (MI) where the heart is subjected to ischemic stress signals such as hypoxia, inflammation and injury, cardiomyocytes increase the secretion of MVs and/or exosomes. In addition, the distinct content between exosomes deriving from the border zone of the MI and the ones originating from the healthy myocardium suggests an adaptive response to injury. Indeed, circulating miRNAs are markedly altered after MI. miR-1 and mir-133 are elevated in the serum of patients with acute coronary syndrome, correlating with the levels of the clinical biomarker troponin T (15). Together with miR-499, these cardiac specific miRNAs are released from the infarcted and peri-infarcted myocardium and regulate the expression of sarcomeric genes and ion channels (15,16). miR-1 is also elevated, together with miR-208, in the urine of acute MI patients (17) indicating that circulating miRNAs released from the injured myocardium can travel to distant organs via exosomes as they are stable and protected from degradation by RNases present in the different body fluids. Many of these miRNAs are released immediately after an insult and could therefore, be used as markers for early detection of acute MI. In fact, circulating miR-126 is an important indicator of damage and repair mechanisms in acute MI patients exemplifying that monitoring of exosomal contents after MI can be a factor of prognosis evaluation and prediction (18,19).

Stem cells have been used in an attempt to regenerate damaged tissue when injected in the injured heart region, by engrafting, proliferating, differentiating and repopulating

the myocardium. The therapeutic effects of stem cells more likely result from the secretion of molecules such as growth factors, antioxidants, cytokines, chemokines and miRNAs with a wide-range of physiological effects (20). The review by Chistiakov *et al.* (14) emphasizes the contribution of cardiac progenitor cells (CPCs) as source of exosomes with regenerative properties. Under hypoxic conditions, CPCs are able to secrete pro-regenerative exosomes capable of inducing tube formation, proliferation and migration of endothelial cells (21,22). Elevated levels of miR-132 and miR-146 have been found in exosomes derived from hypoxic CPCs and infusion of these exosomes in a rat model of ischemia-reperfusion injury reduced fibrosis and enhanced heart function (22). These findings demonstrate how hypoxia is able to re-enforce the regenerative capacity of CPCs via exosomes. Also critical is the type of parent cell secreting exosomes. While delivery of CPCs-secreted EVs to infarcted rat hearts resulted in decreased cardiomyocyte apoptosis, reduced collagen deposition, increased blood vessel density, and ultimately in improved cardiac function, treatment with fibroblast-derived EVs did not have any effect (21). The differences observed may relate to elevated paracrine secretion of miR-210, miR-132 and miR-146a-3p from CPCs and not from fibroblasts (21). Cardiosphere-derived cardiac stem cells were reported to employ a similar mechanism via transfer of miR-146a to mediate cardioprotection by inhibiting apoptosis, promoting cardiomyocyte proliferation and inducing angiogenesis (23). Whether this cardioprotective effect is progenitor/cardiac stem cell preparation-dependent or whether it is mediated by a specific combination of miRNAs and other factors is not clear. Nevertheless, these results suggest progenitor and cardiac stem cells as unlimited sources of cardioprotective exosomes with promising therapeutic potential for post-MI cardiac repair.

Future perspectives/limitations

Only recently, the potential of using exosomes as therapy for MI and other cardiac pathologies has started to be evaluated in preclinical animal models. Among all types of EVs, exosomes are the richest in (micro)RNAs and could constitute a tool for cell- or tissue-specific delivery of RNA molecules of interest after modulating exosomal membrane receptors. One of the main problems in delivering short interfering RNAs (siRNA) or miRNAs *in vivo* is targeting specific tissues and avoiding non-specific delivery. Exosomes, as natural RNA carriers, would also bypass the issue of immunogenicity of siRNA or miRNA or their cargo vehicle. In fact, different strategies have been used to manipulate exosomal content (24) for specific delivery in the brain and targeting of neurons, microglia and oligodendrocytes (25). Exosomes are versatile carriers of both protective and pathological molecular signals and while they appear to be safe and efficient much effort should be directed to identifying their bioactive molecular content as well as understanding how such content will affect specific signaling pathways and cellular mechanisms in recipient cells.

Besides their promising clinical relevance in the identification of novel biomarkers of cardiac injury in ischemic or other cardiac diseases, the clinical and scientific benefits of studying exosomes as extracellular communicators in cardiac disease are multifold. Such studies will reveal novel mechanisms of how cells and organs communicate among each other, aid in understanding and developing new cell therapies for ischemia as well as providing insights for the development of novel therapeutics, and finally to reveal mechanisms of cell targeting for the discovery of novel candidates and delivery of therapeutic compounds for cardiac diseases. But again, issues such as biosafety and tolerance of exosome-based therapies, which could render their translational power, deserve special attention in the future.

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Footnote

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Future Prospect

Is there a role for microRNAs as novel predictors of prognosis in myocardial infarction?

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MicroRNAs (miRNAs) are endogenous, non-coding, single stranded RNA's of 19–25 nucleotides in length that regulate gene expression at the post-transcription processing steps (1). Although the exact biological functions of miRNAs are still not fully understood they have been implicated in cellular development, differentiation, metabolism and death (2,3).

The discovery that stable miRNAs could be identified in human plasma/serum (4) enabled researchers to investigate their expression levels in various cardiovascular conditions (5). Circulating levels of specific miMRAs have been found to be significantly increased in AMI patients compared to controls, leading to speculation that they may have a role to play as novel diagnostic biomarkers (6).

In this edition of gene, Coskunpinar *et al*. present data from a small (27 patients), single centre study, demonstrating the use of circulating miRNA's as novel markers for early prediction of acute myocardial infarction and LV dysfunction post AMI (7). Real time PCR was used to determine the expression levels of 1,116 miMRNs in 27 patients post AMI and 16 control subjects. Six miRNAs were identified as being significantly upregulated in the AMI group compared with the control group.

The most promising miRNA, miR-221-3p was shown to correlate modestly with GRACE and SYNTAX scores as well as serum troponin. In practical terms, the paper suggests that this may represent a novel marker for the early prediction of AMI. However, when miR-221-3p was assessed via receiver operator curves for the prediction of AMI, the AUC was calculated at 0.881, which was inferior to that of the current standard clinical test of troponin (AUC 0.954). Moreover, the addition of miR-221-3p to troponin did not appear to add additional diagnostic capacity.

Ongen *et al*. also observed an inverse relationship with LV ejection fraction. Such relationships would be expected of any biomarker which is collinear with troponin and is therefore not unusual. Moreover, the relationship was demonstrated in uni-variable analysis only and has not been tested in more robust multi-variable analysis.

The observation of relationship between miR-221-3P and LV function is interesting but certainly not a unique finding for miRNA's. Devaux *et al*. (8,9) have previously demonstrated miRNAs to be associated with LV function, remodelling and cardiac contractility post MI in a larger cohort of patients and other studies have shown similar (10).

The utility of miR-221-3p as a prognostic marker has not been tested in this study as no clinical endpoint data were presented. However, one may expect that a biomarker which is associated with Troponin, GRACE and LV function would also be associated with adverse outcomes. Indeed, long term follow up data from the AtheroGene Study identified three miRNAs which precisely predict cardiovascular mortality following AMI establishing their potential as predictors of prognosis (11).

As acknowledged by the authors, the small sample size is the major limitation of this study. Further studies are required both to validate the findings of this small single

centre investigation and also to expand to allow use as a marker of prognosis and adverse LV remodelling. In addition, the authors link the increased expression of miRNAs identified in the study, with the down regulation of four target genes that have previously been found to regulate various cardioprotective molecular pathways. The authors infer that this relationship may hence increase susceptibility to AMI Further research is required in order to establish any potential causative mechanisms.

In conclusion, this study adds to the expanding list of biomarkers in the prediction of AMI. With over 2,000 miRNAs already catalogued (12) this remains a vast topic with many exciting research avenues to pursue in the future. The ability to identify the genetic functions of specific miRNAs and their influence on particular cellular processes significantly increases our understanding of complex pathophysiological mechanisms involved in the development of conditions such AMI.

Combining this knowledge with the rapidly evolving science of genetic engineering is already allowing infections such as Hepatitis C to be tackled in revolutionary ways (13) and may radically change the way we manage numerous cardiovascular diseases in the future.

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Footnote

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Future Prospect

MicroRNAs to take the place of collateral flow index measurements and Rentrop scoring?—Reply to Papageorgiou *et al*.

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We thank Papageorgiou *et al*. for their thoughtful comments on our recent publication regarding circulating microRNAs (miRNAs) associated with coronary collateral artery capacity (1). The benefits of a vast collateral network have been widely accepted to prevent mortality in patients with chronic stable coronary artery disease (CAD) (2,3). Identifying patients with a limited collateral network can in turn distinguish patients at risk to substantial debilitation from adverse cardiac events. Nonetheless, current methods to identify patients with insufficient collateralization are limited to invasive intracoronary collateral flow index (CFI) measurements or angiographic grading (4). Clinical parameters associated with collateral development have been identified (5). However, there remained a lack of biomarkers to discriminate between patients with sufficient or insufficient collateralization.

Genetic heterogeneity in CAD patients has been identified at the messenger RNA level of circulating monocytes and macrophage phenotypes (6,7). This led us to hypothesize that differential miRNA expression in patients with insufficient *vs*. sufficient collateralization must also be present. We identified 4 miRNAs that were significantly upregulated in the plasma of patients with low collateral capacity. We further determined that these miRNAs (miR423-5p, miR10b, miR30d, miR126) could serve as circulating biomarkers to significantly distinguish between chronic total occlusion (CTO) patients with high or low collateral capacity. These miRNAs can discriminate between these patient groups with a positive likelihood ratio between 3.0 and 6.1 depending on the respective miRNA. This revelation can be of immense clinical significance, whereby patients with low collateralization can be potentially identified with a simple blood sample rather than invasive intracoronary catheterization. However, our study warrants further studies with larger patient cohorts, along with examining the utility of these miRNAs as biomarkers for collateralization in CAD patients.

Papageorgiou *et al*. point out that the utility of these miRNAs is also dependent on a generally accepted definition of low collateral capacity, whereby some studies use Rentrop scoring and others use different thresholds of pressure-derived CFI (CFI_p) to establish the level of collateralization. Rentrop scoring provides only a semi-quantitative measure and is limited to collateral vessels above 100 μm diameter. In relation to CFI_p measurements, it is important to recognize the difference between CAD and CTO patients, whereby CTO patients provide no variability in coronary lesion severity as compared to CAD patients. As a result, the distribution of CFI_p measurements in CTO patient populations differs from that in CAD patients. The mean CFI_p in a cohort of 295 patients was deemed to be 0.39 (5), while in CAD patients the frequency distribution of CFI_p is dependent on the severity of CAD (2).

As both Papageorgiou *et al*. and we have mentioned, a number of co-existing parameters may affect the diagnostic ability of miRNAs in the general population. Medication

usage, gender, age as well as diabetes mellitus have been shown to affect both collateral vessel development as well as miRNA expression levels (8-11). In our study, gender and age significantly impacted the predictive power of the respective miRNAs as suitable biomarkers. However, miR126 demonstrated significant predictive power to discriminate between patients with high and low collateral capacity even without consideration of age and gender. MiRNA126 has been largely linked to angiogenesis, as well as atherosclerosis, whereby the mature miRNA-126-5p plays a role in endothelial turnover (12). Examining larger patient cohorts will likely elucidate the exact threshold of the respective miRNA expression levels that can distinguish between these patient groups, and provide additional insight in the effects of other co-existing parameters on this threshold.

In conclusion, circulating miRNAs offer a new method for patient stratification. Nonetheless, there still remains a large step before miRNA examination can become a part of routine clinical application. As the possibility to distinguish the coronary collateral artery capacity of patients without the need for invasive catheterization is of great clinical significance, our results warrant further investigation in larger CTO patient cohorts, along with examining the predictive power of these miRNAs in CAD patients.

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Footnote

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Future Prospect

Circulating extracellular vesicles containing miRNAs may have utility as early biomarkers for cardiac injury

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Through advancements in prevention, awareness, detection, and treatment modalities, rates of myocardial infarction (MI) have decreased over time. However, MI still remains a significant cause of morbidity and financial burden within the United States, with approximately 3.2 per 1,000 individuals hospitalized per year, corresponding to an estimated 11.3 billion dollars in societal costs (1). The universal definition of MI specifies that patients must have a rise and fall of cardiac biomarkers as well as ischemic symptoms for diagnosis. Currently, circulating biomarkers in plasma including creatinine kinase MB (CK-MB) and cardiac troponin remain the gold standard for detection and validation of type 1 MI. Although assays using high sensitivity troponin are able to detect MI as early as 2–3 hours after cardiac injury with good sensitivity and specificity (2), in certain patients the initial assay may be negative. Because early recognition of MI is associated with improved outcomes, decreased hospital length of stay, and decreased cost (3-6), there is interest into novel diagnostic methods for MI. Beyond diagnosis of MI, improving the detection and etiology of myocardial injury using novel biomarkers may enhance the management of a variety of cardiac conditions.

It is in this context that Deddens and coauthors (7) present a study investigating the possible utility of quantification of microRNAs (miRNAs) and extracellular vesicle (EV) release to assist in early determination of myocardial injury. Using mouse and porcine models, their study demonstrates that circulating EVs as well as miRNAs are significantly increased in animals with induced MI as compared with sham controls early after ischemia. The authors demonstrate that after ligation of the LAD in mice to induce myocardial ischemia, followed by reperfusion (n=3), the amount of EVs released are significantly greater than the sham arm (n=1) in mice at 150 minutes. Moving to porcine models, the authors serially sample plasma to determine the level of circulating miRNA at different time points. They demonstrate that the circulating miRNAs previously demonstrated to be increased in plasma after MI in humans are also increased in animal models with a significant increase demonstrated in cases as compared to controls 2.5–3.5 hours after ischemia. The authors found that significantly elevated miRNAs include miRNA-1, -208, and -499 but not miRNA-21 or miRNA-146a, and that these levels are higher in EV than in plasma.

Although limited by small study size, the study offers interesting opportunities for translational medicine. Because miRNAs are involved in gene expression at a post translational level, the ability to understand the role of miRNA in pathological processes may also provide possible therapeutic targets (8). Although the purpose of the analysis by Deddens and colleagues was not to determine the exact mechanism and significance of miRNA elevation after MI, identification of key miRNAs after ischemia is an important step in better understanding the physiologic process that occurs when myocardium becomes ischemic.

The importance of miRNAs in the post transcriptional regulation of gene expression is increasingly recognized (9). Prior studies have identified certain miRNAs as markers of cardiac ischemic/reperfusion injury with both regulatory,

protective, and diagnostic utility (10-12). In addition to modifying myocardial gene expression in response to injury, miRNA are secreted in a regulated manner into the circulation by EVs as part of intercellular communication (similar to hormones). The presence of EVs in the circulation provides an important "window" into the injured myocardium that is otherwise inaccessible in the clinical setting. EVs package miRNA in specific proteins (e.g., Ago2 or HDL) which render miRNA highly resistant to degradation. Unlike most extracellular RNA which is rapidly degraded in the absence of RNAse inhibitors or strict handling conditions, EV miRNA is robust to degradation under most conditions. This feature coupled with the ability to readily measure RNA in clinical laboratories make EV miRNA an attractive platform for a clinical biomarker.

In addition to detection of myocardial necrosis, miRNA quantification and assessment may be of utility in elucidating the mechanism of myocardial injury, assisting in the prognostic and diagnostic capabilities in acute MI. This highlights possible future avenues of translation into clinical practice, with possible benefit in distinguishing between MI, heart failure, myocarditis, and other processes involving myocardial injury upon patient presentation. Additional potential applications include myocardial monitoring for cardiac allograft rejection, chemotherapy induced cardiomyopathy, asymptomatic severe valvular disease, and risk stratification during exercise stress testing.

Excitingly, miRNAs are also emerging as potential therapeutic targets, thus better understanding of their utility, function, and targets are a priority for investigation (13). EV miRNA are also being developed as therapeutic targets as the extent of their increase has been shown to be associated with worsened myocardial injury (14). Studies have demonstrated improvement in hepatitis C viremia in primate studies (15) and colon cancer (16), however the full potential of miRNA as therapeutic agents is only beginning to be fully realized.

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Future Prospect

Long non-coding RNAs in heart failure: a promising future with much to learn

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El Azzouzi and colleagues (1) recently published a perspective describing our work, in which we characterised the murine long noncoding RNA (lncRNA) transcriptome post myocardial infarction (MI) (2). This perspective nicely summarised key aspects of our work. However, we would like to respond with some additional comments to clarify and expand important points resulting from their perspective and our work.

Considering the emerging roles for lncRNAs in dictating the gene regulatory networks underpinning developmental and disease processes (3,4), we set out to characterise this landscape within the infarcted mouse heart (2). We utilised an extremely deep RNA sequencing approach coupled with *de novo* transcript reconstruction to comprehensively profile and discover novel lncRNAs. As discussed, we identified approximately 1,500 previously unannotated lncRNAs, with a significant fraction being differentially expressed post stress. Importantly, through integrating publicly available transcriptomic and epigenomic datasets, we demonstrated that our newly discovered lncRNAs exhibited some unique functional and regulatory characteristics. Firstly, they were significantly more enriched in the heart as compared to protein coding genes (PCGs) or previously annotated lncRNAs. Much of this increased heart specificity is likely as a consequence of their functional genomic loci of origin that are active cardiac enhancer regions. Enhancers are important distally acting cis regulatory sequences (5-7). They are considered the key information processing units within the genome integrating temporal and spatial cues to direct correct context-specific gene expression patterns. Thus, we found that the novel lncRNAs that were modulated post-MI were significantly more associated with active cardiac enhancer sequences, therefore implicating the enhancer landscape and their associated lncRNAs, in the transcriptional reprogramming that underpins pathological remodelling. Finally, and importantly from a translational and therapeutic perspective, we identified hundreds of predicted human orthologs and validated their expression in human samples of a pathological nature (8).

Despite the insightful perspective by El Azzouzi *et al.* (1), we would like to emphasise and clarify a few points. Firstly, what was somewhat unique and surprising during our initial analysis was the sheer number of novel lncRNAs we were able to discover. This, as we have discussed in depth elsewhere (9) and would like to emphasise here, is as a direct consequence of the RNA sequencing depth. We would strongly argue based on our previous experience, that sequencing to a depth of at least 300 million paired-end reads is essential for the identification of novel tissue- and context-specific lncRNAs. With this sequencing depth, we were able to identify the 1,500 novel lncRNAs described in this study but also 2,500 novel lncRNAs in mouse embryonic stem cells (ESC) differentiating towards cardiomyocytes (10). To support our argument, we performed a computational simulation, in which we counted how many novel lncRNAs would have been discovered at different sequencing depths. We found that if we would have sequenced to the widely used depth of

50 million paired-end reads, we would have not detected approximately 50% to 60% of novel lncRNAs in both our studies (9). Strikingly, one should consider that the novel lncRNAs that required the greatest depth of sequencing for discovery corresponded to those exhibiting the most interesting regulatory and functional characteristics, in particular heart-enriched lncRNAs associated with cardiac developmental and functional roles. Those lncRNAs that were discovered at the shallower sequence depth were typically already annotated housekeeping like and non-tissue enriched lncRNAs.

El Azzouzi *et al*. (1) highlighted the cardiac-enriched nature of our novel lncRNAs. Despite validating a number of them via qPCR, it is important to note that this conclusion was primarily based on the integration of publicly available tissue-specific RNA-seq datasets. By using ENCODE data, we quantified the expression of every transcript in the heart and 17 non-cardiac tissues (2,9). Heart specificity was then determined for each transcript by calculating the expression in the heart versus the mean expression in all 17 non-cardiac tissues, generating a specificity score for each transcript (9). This approach is powerful and has been successfully used for lncRNAs discovered in a number of cell types and tissues demonstrating that newly discovered lncRNAs are typically more tissue-enriched *vs*. PCGs and also miRNAs (11). This is of important therapeutic potential, as targeting cell- and tissue-restricted lncRNAs could increase the specificity of future therapies and reduce off-target side effects. These observations also support the notion that lncRNAs could represent highly specific biomarkers for pathological processes (11,12). Indeed, a number of recent studies are beginning to validate this hypothesis. Along the same line, we demonstrated that cardiac lncRNAs expression is extremely well correlated with electrocardiographic traits. It is important to emphasise here that this correlation was executed in mouse and not in human patients as mentioned by El Azzouzi *et al*. (1). Comparable observations have however been made in human patients (11,12), supporting our work in mice. Furthermore, a number of recent studies have demonstrated the presence of lncRNAs circulating in the plasma (12). The detection of cardiac-enriched lncRNAs in the plasma therefore warrants interrogation and promises to be an exciting future application of our work with respect to post infarction remodelling and heart failure.

Despite the relative ease in discovering lncRNAs implicated in cardiovascular development and disease, elucidating their functions remains a major challenge. We applied a novel approach to infer functions for lncRNAs based on chromatin state transitions during the differentiation of ESCs to cardiomyocytes (2,9). We believe this approach represents a relatively simple method to begin to prioritise cardiovascular lncRNAs for functional characterisation. The analysis is based on the observation that PCGs in differentiating cells cannot be assigned to a particular function based on their expression kinetics alone. However, Wamstad *et al*. (13) found using genomewide epigenomic profiling that PCGs co-expressed during cardiac differentiation can be functionally grouped based on distinct chromatin state transitions at their promoters. More specifically, they concluded that sub-groups of co-expressed PCGs, clustered based on unique chromatin state transitions, were involved in highly specific and distinct biological processes pertinent to cardiac development and function. These biological processes included cardiac signalling, metabolism, development and muscle contraction. We therefore surmised that novel lncRNAs sharing these unique chromatin state patterns at their promoters were likely to be involved in parallel biological processes. This approach therefore provides a very powerful and unbiased proxy to infer functions for novel lncRNAs. Supporting this, we found that heart-enriched novel lncRNAs were preferentially associated with clusters linked to fundamental cardiac biological processes including cardiac contraction and development. Finally, we also used this approach to predict targets of novel lncRNAs based on shared chromatin state patterns. For example, we demonstrated that Novlnc6 was able to regulate Bmp10, which shared the same chromatin patterns during differentiation. This resulted in a specific impact on the key core cardiac transcription factor, Nkx2-5, a known target of Bmp10 (2).

To conclude, our work, in addition to a number of other studies characterising the long noncoding transcriptome in disease (14,15), opens a new era of vast therapeutic and diagnostic potential. This highly integrated layer of noncoding RNAs exhibits interactions with components of various effector complexes to dictate the activity of developmental and pathological cardiac gene regulatory networks. Our data clearly demonstrates that pathological states within the heart are intrinsically linked to the dynamics of lncRNA expression. In the future, it will be of critical importance that investigators discovering novel cardiovascular lncRNAs leverage both computational and experimental approaches to functionally characterise the landscape of lncRNAs within the heart (9). In particular, one should focus on deciphering the mechanisms and language

through which lncRNAs interact with their respective protein, RNA or DNA partners. These approaches should illuminate our understanding of these very exciting therapeutic and diagnostic molecular targets.

Acknowledgements

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Footnote

Conflicts of Interest: The authors have filed a patent about the diagnostic and therapeutic use of several heart enriched lncRNAs.

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Future Prospect

Long non-coding RNAs in heart failure: an obvious lnc

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Provenance: This is a Guest Perspective commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Abstract: Heart failure is a life-threatening and costly ailment characterized by structural and functional impairment of the heart. Despite major advances in understanding protein-mediated transcriptional control and signaling pathways that underlie the cellular and interstitial alterations of heart failure, significant therapeutical breakthroughs for innovative treatments of this disease are still missing. The recent extensive profiling of the mammalian transcriptome has revealed a large number of long non-coding RNAs (lncRNAs) that play a diversity of important regulatory roles in gene expression. In here, we focus on a recent work by Ounzain and colleagues comprising genome-wide profiling of the cardiac transcriptome after myocardial infarction with an emphasis on the identification of novel heart-specific lncRNAs.

Keywords: Long non-coding RNA (lncRNA); heart failure; gene networks

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Heart failure is a life-threatening and costly condition categorized by structural and functional impairment of ventricular filling or ejection of blood by the heart. Considering the overall increase in lifespan of the general population and high incidence of risk factors and improved survival from acute cardiovascular events, it is expected that the prevalence of heart failure will increase along with the associated emotional and socio-economic burdens. Upon injury, the heart responds to a diversity of biochemical and hemodynamic stressors through a process that entails molecular, cellular and interstitial alterations that are reflected further in size, shape and function of the heart (1). Although the initial response is adaptive and mainly serves to balance the decay in the pumping capacity of the heart, prolonged stress stimulation progressively contributes to deteriorate ventricular function which ultimately can lead to heart failure. Despite major advances in understanding the mechanisms that underlie cardiac remodeling and the worsened cardiac performance, this did not translate into significant therapeutical breakthroughs for innovative treatments of heart failure in patients.

Once considered transcriptional noise, several transcriptome studies have shown prevalent transcription of a large number of non-coding RNAs (ncRNAs) (2). These ncRNAs are classified into two groups according to their length, whereby small ncRNAs are arbitrarily set at less than 200 nucleotides in size and include several ncRNAs species including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs) (3-5). In addition, transcripts of ncRNAs that are larger than 200 nucleotides are called long non-coding RNAs (lncRNAs). Of all currently studied noncoding genes, lncRNAs are a mystery species of growing scientific interest. Just like the extensively studied miRNAs, lncRNAs have been reported to have divers biological functions such as regulation of chromatin remodeling, maintenance of the nuclear structure

integrity, and transcriptional and post-transcriptional processing (6,7). Despite their length , lncRNAs are distinct from protein coding genes as they are expressed at lower levels, are less evolutionarily conserved and mainly consist of a distinctive gene structure of usually 1–2 exons (8,9). In contrast to other ncRNAs, some lncRNAs have been shown to give rise to small peptides (10), showcasing the diversity of lncRNAs. The importance is underlined by the fact that specific lncRNA knockout strategies in mice showed severe effects that caused pre- and postnatal lethal phenotypes (11,12). Whereas deletion of lncRNAs *Peril* and *Mdgt* resulted in viability defects, mice lacking the lncRNA *Fendrr* died at the embryonic stage due to defects in several organs including the heart (11). These developmental defects were shown to be independent of adjacent genetic regions, e.g., *cis* or enhancer-like regulatory domains, as deletion of these lncRNAs did not phenocopy deletion of adjacent genes, which suggests a distinct function for these lncRNAs (12). One of the first reports on ncRNA expression showed that infants with non-syndromic tetralogy of Fallot had higher levels of an ALC-1 antisense lncRNA that disturbed ALC-1 translation in hypertrophied ventricles compared to their healthy counterparts (13,14). Next to the fact that some lncRNAs have been found to be altered in the developing or diseased heart, several single nucleotide polymorphism (SNP) in lncRNAs have shown to be strongly correlated with cardiovascular disease (15,16). For example, SNPs in myocardial infarction associated transcript (MIAT) and antisense non-coding RNA in the INK4 locus (ANRIL) forecast increased risk of cardiovascular disease (15,16). Moreover, lncRNA H19 was significantly upregulated in failing murine hearts, suggesting a role for hypoxia regulated lncRNA expression in heart failure (17). This is further highlighted by a recent study employing a comprehensive myocardial transcript profiling of coding and non-coding RNA species in failing human hearts and hearts that were unloaded with a left ventricular assist device (LVAD) (18). LVAD support has been known to diminish cardiac stress by reducing tissue hypoxia, auxiliary to the concept of restoration of cardiac normoxia by mechanical unloading (19,20). Fascinatingly, a significantly higher proportion of differentially expressed lncRNAs was normalized in hearts that were unloaded compared to mRNA and miRNA levels (18). In fact, lncRNA levels not only responded more sensitively to LVAD support but their expression profile allowed to distinguish left ventricular samples from patients with ischemic and non-ischemic heart failure before and after LVAD support (18). It is no longer a question whether lncRNAs have a role in the cardiovascular system under normal physiological condition and in disease states. However, understanding the comprehensive role of lncRNA played herein is still in its infancy.

In an attempt to elucidate this, Ounzain and colleagues report an exciting genome-wide profiling of the cardiac transcriptome after myocardial infarction with an emphasis on the identification of heart-specific lncRNAs (21). In the article by Ounzain *et al.*, the authors performed RNA sequencing, ab initio transcript reconstruction and integrated genome-wide approaches to supplement gene regulatory networks with lncRNA profile in hearts after myocardial infarction. Of the multi-exonic transcripts, 14.3% were allocated as lncRNAs of which a smaller part (5.6%) corresponded to the UCSC annotation and a larger part (8.7%) was still not annotated. These unannotated transcripts showed several typical lncRNA physiognomies, such as genomic orientations relative to nearby coding genes and the low protein coding potential, and were therefore designated as novel lncRNAs. To verify whether these novel lncRNAs are cardiac specific and are associated with enhancer sequences active in the heart, qRT-PCR verification and association with flanking *cis*-regulatory proximal coding genes showed that differentially expressed novel lncRNAs were significantly more enriched in the myocardium than other transcript classes. Intriguingly, although gene ontology classification of the proximal genes revealed enrichment in processes associated with cardiac function and transcriptional regulation, novel lncRNAs were significantly associated with transcriptional control. Moreover, these lncRNAs were also located proximal to cardiac developmental genes, implicating a role for these novel lncRNAs in the stress responses and transcriptional reprogramming of the remodeling heart. Indeed, it was suggested that lncRNAs expression profiles in general are more profound to different heart failure etiologies when compared to other RNA transcripts (18). Mechanistically, Ounzain *et al.* demonstrated in the current study that the lncRNAs act as competitive endogenous RNAs specifically for microRNAs. Correlation network analysis of miRNA-lncRNA-mRNA indicated that lncRNAs correlated positively with miRNAs that have been known to be

modulated in the post-infarcted myocardium, whereas mRNA levels correlated negatively. The likelihood that the predominant mechanism of action of cardiac lncRNAs is *cis*-, rather than *trans*-, gene regulation implicates a role as decoys for miRNAs. This is further exemplified by a study showing that linc-MD1, a muscle-specific lncRNA, absorbs miR-133 to regulate the expression of transcription factors that activate muscle-specific gene expression such as MAML1 and MEF2C (22). Moreover, linc-MD1 correlated strongly with aberrant muscle loss, as is seen in patients with Duchenne muscular dystrophy (23). In a cardiac setting, Wang *et al.* reported that cardiac apoptosis-related lncRNA (CARL) indirectly regulated mitochondrial fission and apoptosis, via an endogenous miR-539 sponge and thereby regulates PHB2 expression (24).

To assess whether the cardiac transcriptome, in particular the novel lncRNAs, correlated with the contractile and remodeling parameters of the heart *in vivo*, the cardiac transcriptome was associated with physiological traits in human patients. Though the novel reported lncRNAs correlated better with all physiological traits assessed, clustering for both coding transcripts and novel lncRNA indicated a group of related lncRNAs that correlated positively with cardiac function and negatively with remodeling parameters. The complexity to delineate how these particular physiological traits contribute to cardiac function and the function of lncRNAs herein is underscored by a single cluster showing diverse correlation to these interlinked functional parameters. Moreover, determining critical regulatory elements of transcriptional activity such as distinct chromatin states and methylation and acetylation of histone lysine residues is a major advance in understanding transcriptional regulation of lncRNAs. In line, although the novel lncRNAs were associated with more active chromatin states, their expressions showed to be more dependent on a canonical promoter signature rather than an exclusively active enhancer signature.

Although the lncRNAs complied with the UCSC annotation and have typical lncRNA physiognomies, validation of these transcripts is necessary to filter out false reads. The authors used qRT-PCR to validate a select group that was associated with functional parameters and to determine timely and regional cardiac expression. In contrast to mRNAs, lncRNA themselves must physically localize to their particular site of action, making knowledge of lncRNA subcellular localization patterns vital for insights into their biology and function. Nuclear and cytoplasmic fractionation could indicate subcellular enrichment of an lncRNA and give insights into the functionality of the lncRNA. Indeed, an exclusive nuclear localization would argue against putative short peptide sequences encoded by the lncRNA, since translation occurs in the cytoplasm. However, to fully delineate the exact role of an lncRNA, further analysis on localization to particular subcellular areas within the cytosol or nucleus may lead to better insights on the role of an lncRNA. For example, lncRNAs such as NEAT1 and MALAT1 have been shown to localize to nuclear bodies within the nucleus and the lncRNA GAS5 was shown to shuttle between the nucleus and cytoplasm (25,26). More elegantly, Clemson and colleagues showed that the lncRNA XIST specifically accumulates on the inactive X-chromosome (27). Hence, RNA fluorescence *in situ* hybridization (RNA FISH) could better address these questions and reveal potential mechanisms for the described novel lncRNAs. Moreover, this technique would directly discriminate the cell type presence *in situ* of these novel lncRNA in the injured myocardium instead of the laborious and indirect detection in isolated different cell types

For a long time heart function was considered to be orchestrated through regulatory networks that are composed solely of a protein-mediated transcriptional control and signaling pathways, and which were perturbed during cardiac disease. In line, efforts for therapeutical interventions were systematized around these networks with little translational outcomes so far. Clearly the path of lncRNAs from background noise and serendipitous transcription to key regulators has led to significant insights into gene regulatory networks (*Figure 1*). This previously hidden layer of gene regulation underpins the sophisticated regulatory networks to not only coordinate physiological responses and environmental adaptation, but also pathophysiological responses in multifaceted heart diseases. In order to take the next step towards modulation of lncRNA levels as an approach for tackling cardiac disease, reviewing their function and role in cardiac biology is of utmost importance. Therefore, efforts to unveil the multiple lncRNAs and their role in cardiac disease, exemplified by the study of Ounzain and colleagues, provides an excellent toolkit to assist in exploring the possibilities for ranging from biomarkers of disease to targets for therapeutical intervention.

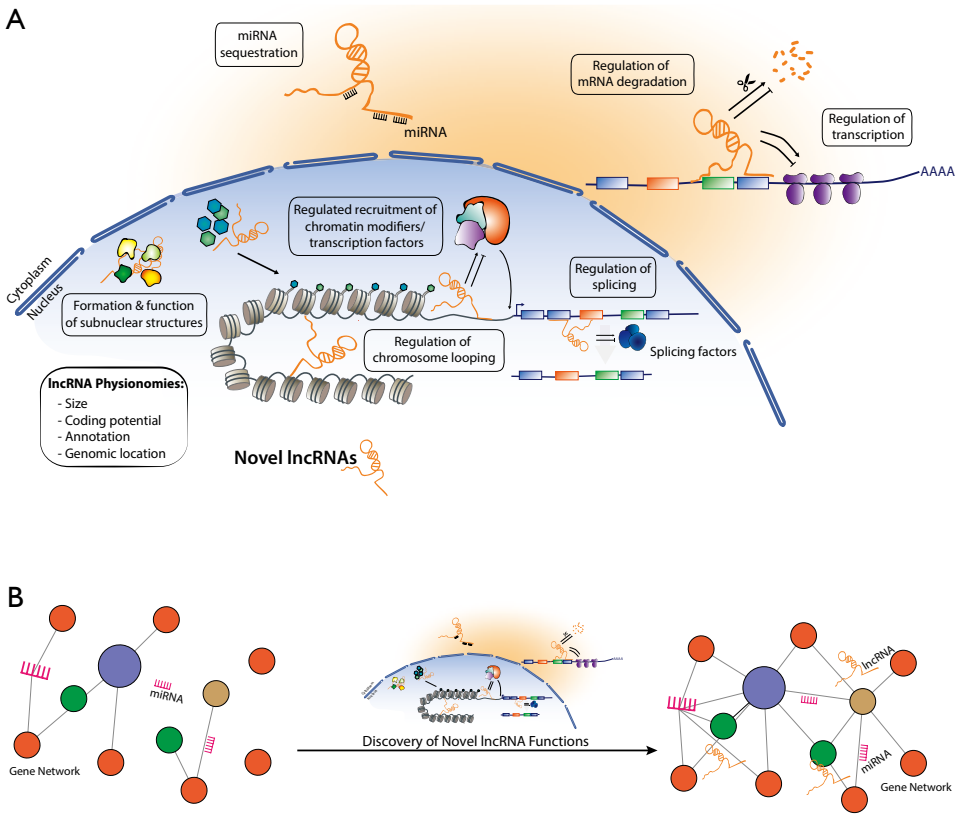

Figure 1 Long non-coding RNAs (lncRNAs) and their functions. (A) Possible functional mechanisms for the novel lncRNAs; (B) lncRNAs as an additional layer of controllers of gene regulatory networks.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Future Prospect

Association between microRNAs and coronary collateral circulation: is there a new role for the small non-coding RNAs?

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Provenance: This is a Guest Editorial commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Hakimzadeh N, Nossent AY, van der Laan AM, *et al.* Circulating MicroRNAs Characterizing Patients with Insufficient Coronary Collateral Artery Function. PLoS One 2015;10:e0137035.

Abstract: We read with interest the article entitled "Circulating microRNAs characterizing patients with insufficient coronary collateral artery function" which was recently published in the PLOS ONE journal. It was demonstrated for the first time that specific circulating microRNAs (miRNAs) can distinguish patients with sufficient from those with insufficient coronary collateral circulation. Circulating miRNAs in the plasma of patients with stable CAD and chronic CTO could provide information with regard to the coronary collateral artery capacity. However, several aspects need to be taken into consideration before the use of miRNAs in the clinical practice. A risk model that would incorporate risk factors for cardiovascular disease and miRNAs could prove to be very useful. Although an association between the levels of miRNAs and the collateral artery capacity appears promising, it still does not confirm any causal role for miRNAs. Therefore, large clinical studies in populations with CTO are warranted to evaluate this finding.

Keywords: microRNAs (miRNAs); coronary artery collaterals; angiogenesis

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Studies have shown that the presence of sufficient collateral circulation is beneficial for patients with stable CAD, as it improves the survival rates (1). A circulating biomarker that would provide us with the appropriate information regarding to the existence of collateral circulation in these patients would be very helpful in the clinical setting. microRNAs (miRNAs) actively participate in cardiovascular homeostasis and play an important role in the initiation and progression of cardiovascular disease (2). Recent data suggest that miRNAs contribute to the formation of vulnerable atherosclerotic plaques (3), while they enhance angiogenesis as well. Therefore, it has been speculated that circulating miRNAs can provide information about the collateral artery network of patients with chronic total occlusion (CTO). In the present article, Hakimzadeh *et al.* (4) showed for the first time that specific circulating miRNAs can distinguish patients with sufficient and patients with insufficient coronary collateral circulation.

miRNAs and angiogenesis

It was an interesting discovery that the enzymes involved in miRNAs maturation also participate in angiogenesis: Dicer inhibition resulted in a decrease in angiogenesis *in vivo*, while inhibition of Drosha resulted in an anti-angiogenic effect *in vitro* (5). Since then, studies have shown that several miRNAs upregulate angiogenesis, while others suppress angiogenic pathways (6) (*Figure 1*). However, the data were mainly derived from preclinical studies, until Nie *et al.* (7) showed that miR-126 levels, along with vascular endothelial

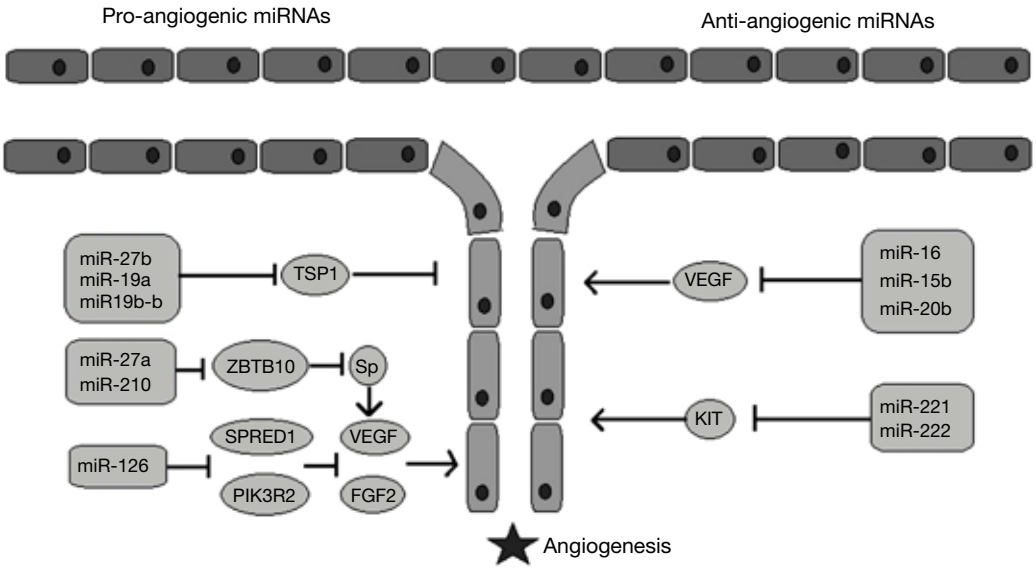

Figure 1 Pro-angiogenic versus anti-angiogenic miRNAs. Some miRNAs upregulate angiogenesis by promoting angiogenic factors (i.e., VEGF) or by inhibiting anti-antiogenic mediators (i.e., TSP1), while others downregulate angiogenesis by inhibiting anigiogenic molecular pathways. VEGF, vascular endothelial growth factor; TSP1, thrombospondin 1; ZBTB10, zinc finger and BTB domain-containing protein 10; SPRED1, sprout-related EVH1 domain-containing protein 1; PIK3R2, phosphatidylinositol 3-kinase regulatory subunit beta; Sp, substance P; FGF2, fibroblast growth factor; KIT, receptor kinase protein/gene.

growth factor (VEGF) levels, were higher in healthy people and in patients with well-developed collateral arteries compared to patients with under-developed collateral circulation. In addition, miR-126 levels could independently predict coronary collateral circulation formation. Nevertheless, Hakimzadeh *et al.* (4) provided further insight to this issue. Their study included patients undergoing successful percutaneous coronary intervention and had CTO of a coronary artery. The levels of miR 423-5p, miR-30d, miR-10b and miR-126 were increased in the setting of insufficient coronary collateral artery capacity. In comparison to healthy controls, though, only the levels of miR-30d and miR126 were found to be elevated. Indeed, there is evidence that gene modulation can discriminate between patients with well developed and patients with poorly developed collateral arteries. van der Laan (8) showed in patients with CTO that the mRNA expression of galectin-2 was increased in monocytes of patients with low collateral flow index (CFI). The rs7291467 polymorphism was associated with increased galectin-2 levels and a lower angiogenic response. Nevertheless, the role of miRNAs in angiogenesis was only recently examined.

Several studies have proved the angiogenic potential of miR-126. A study that included patients with right ventricular heart failure and pulmonary hypertension showed that lower levels of miR-126 were expressed in right ventricular tissues of patients with decompensated heart failure. Of note, this was associated with decreased capillary density. The *in vivo* upregulation of miR-126 improved vascular density in an experimental animal model of pulmonary artery hypertension (9). More recently, the administration of miR-126 through ultrasound-targeted microbubble destruction resulted in increased vascular density in an animal model of chronic ischemia, as it enhanced VEGF and promoted angiopoietin-1 signaling (10).

In turn miRNA-10b has been shown to regulate angiogenesis in glioblastoma multiforme (11) and it was found to be implicated in vascular smooth muscle cell proliferation which is associated with atherosclerosis progression (12). miR-423-5p has been recently recognized as a novel biomarker for congestive heart failure and correlates with pro-brain natriuretic peptide (pro-BNP) levels. As opposed to miR-30d, though, miR-423-5p has been linked to increased cardiomyocyte apoptosis (13).

miRNAs as circulating biomarkers for coronary collateral circulation: challenges to meet

A circulating biomarker that would determine patients with poorly developed collateral coronary artery network would be

Table 1 Selected studies investigating predictors for poor coronary collateral capacity

Studies	Year	Population	N	Collateral flow stratification	Result
Hakimzadeh *et al.* (4)	2015	CTO patients undergoing coronary angiography	41	Poor CCC: CFI <0.39 Good CCC: CFI >0.39	(+) miRNA-126, miRNA-10b, miRNA-30d and miRNA-423-5p in patients with poor CCC
Nie *et al.* (7)	2014	CAD patients with ≥95% stenosis in a coronary artery	120	Poor CCC: grade 0 and grade 1 Rentrop Good CCC: grade 1 and grade 2 Rentrop	miR-126 and VEGF levels independently predicted CCC development
İleri *et al.* (15)	2016	Patients with NSTEMI	224	Poor CCC: grade 0 and grade 1 Rentrop Good CCC: grade 1 and grade 2 Rentrop	DM, WBC, neutrophil counts and NLR independently predicted low CCC; age negatively predicted poor CCC
Kalkan *et al.* (16)	2014	Patients with CTO	274	Poor CCC: grade 0 and grade 1 Rentrop Good CCC: grade 1 and grade 2 Rentrop	NLR, hs –CRP, WBC independently predicted poor CCC;
Baykan *et al.* (17)	2015	Patients with CTO	163	Poor CCC: grade 0 and grade 1 Rentrop Good CCC: grade 1 and grade 2 Rentrop	(+) AIx, PWV, fasting glucose, creatine, uric acid, neutrophil count and NLR in patients with low CCC
Yetkin *et al.* (18)	2015	Patients with at least one coronary stenosis of ≥95% that underwent coronary angiography	502 (228 with CTO)	Poor CCC: grade 0 and grade 1 Rentrop Good CCC: grade 1 and grade 2 Rentrop	DM and female gender predicted poor CCC; monocyte count was independent of CCC
van der Hoeven *et al.* (19)	2013	Patients with CTO	295	Poor CCC: CFI <0.39 Good CCC: CFI >0.39	Beta blockers, hypertension and angina pectoris were positively associated with CFI; WBC, prior MI and high DBP were negatively associated with CFI
van der Laan *et al.* (8)	2012	Patients with CTO	50	Dichotomized according to CFI	(+) mRNA expression of galectin-2 in monocytes of patients with poor CCC; (+) polymorphism rs7291467 CC genotype in patients with poor CCC

(+), increased. CCC, coronary collateral circulation; CTO, chronic total occlusion; CFI, collateral flow index; CAD, coronary artery disease; VEGF, vascular endothelial growth factor; NSTEMI, non ST-elevation myocardial infarction; WBC, white blood cell; NLR, neutrophil to monocyte ratio; hs-CRP, high sensitivity CRP; AIx, augmentation index; PWV, pulse wave velocity; MI, myocardial infarction; DBP, diastolic blood pressure.

of great clinical significance, since the invasive procedure of coronary angiography could potentially be avoided. In addition, coronary angiography can disclose arteries of >100 μm (14), thus cannot provide insight into microcirculation. It appears that the findings of Hakimzadeh *et al.* (4) are promising. However, up to date, no equivalent circulating biomarker exists (*Table 1*). That makes it difficult to compare the discriminatory capacity of miR 423-5p, miR-30d, miR-10b and miR-126 with the discriminatory capacity of an established circulating biomarker. It should be mentioned that a generally accepted definition of low collateral capacity is necessary, so that the results of the future studies will be comparable. For example, Nie *et al.* (7) used Rentrop grades to assess coronary collateral circulation, while other studies (1) considered collateral circulation capacity to be insufficient when CFI was <0.25. Hakimzadeh *et al.* (4) defined insufficient collateral circulation as the one with CFI<0.39 and this was in agreement with the study of van der Hoeven *et al.* (19). It should be stressed out that the intracoronary assessment of CFI in healthy individuals

is not feasible, thereby limiting the information about the collateral network in this population. Despite this limitation, the levels of miR-30d and miR-126 were found to be lower in healthy individuals compared to patients with CTO (4).

Several co-existing parameters might affect the diagnostic ability of miRNAs in the general population. Diabetes mellitus interferes with the formation of collateral coronary artery network (15), while it has been found to down regulate the expression of several miRNAs, including miR-126 (20). A recent risk scoring model that predicts poor collateral coronary circulation showed that a combination of white blood cell count, age and history of myocardial infarction can predict poorly developed collaterals (15). Therefore it is necessary to stratify patients' risk for poor collateral coronary circulation before attempting to evaluate the levels of a circulating biomarker. In the present article (4), patients with diabetes mellitus and a history of myocardial infarction were excluded from the study so that the expression patterns of miRNAs could be examined independently. Of note, the discriminatory power of miR-10b, miR-30d, miR-423-5p was evident only after multivariate analysis, which underscores the significance of taking all clinical parameters into consideration. Since leukocytes were found to be implicated in collateral artery growth (15), their assessment seems mandatory. Hakimzadeh *et al*. (4) found an association between miRNA-10b levels and monocyte/leukocyte count, suggesting a possible link between these parameters. Finally, aspirin administration has been found to decrease miR-126 levels, since platelets are a major pool of circulating miR-126 (21). Nevertheless, in the present article, aspirin administration did not blunt the discriminatory efficacy of miR-126 (4).

Conclusions

Circulating miRNAs in the plasma of patients with stable CAD and CTO have been shown to provide information about the coronary collateral artery capacity. This could possibly suggest an alternative diagnostic route to the invasive coronary angiography. However, several aspects need to be taken into consideration before the use of specific miRNAs could be applied into clinical practice. Co-existing parameters, such as diabetes mellitus and leukocyte count, affect angiogenesis and might interfere with the diagnostic efficacy of miRNAs; therefore, a risk model that would incorporate such parameters could be useful. It has become evident that an association between the levels of circulating miRNAs and the collateral artery capacity is not enough to confirm an underlying causative mechanism. Therefore, many more large clinical studies in populations with CTO are warranted to evaluate this finding.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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