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ARTYKUŁ SPECJALNY / *STATE-OF-THE-ART REVIEW*

MicroRNAs: powerful regulators and potential diagnostic tools in cardiovascular disease

Dominika Klimczak[1], Leszek Pączek[2], Krystian Jażdżewski[3, 4], Marek Kuch[1]

[1]Division of Heart Failure and Cardiac Rehabilitation, Department of Cardiology, Hypertension and Internal Medicine, Second Faculty of Medicine, Medical University of Warsaw, Warsaw, Poland

[2]Department of Immunology, Transplantology and Internal Diseases, Medical University of Warsaw, Warsaw, Poland

[3]Genomic Medicine, Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Warsaw, Poland

[4]Laboratory of Human Cancer Genetics, Centre of New Technologies, CENT, University of Warsaw, Warsaw, Poland

INTRODUCTION

Initial sequencing of the human genome revealed that only 1.5% of human DNA codes for proteins [1]. The available evidence demonstrates that a significant fraction of the remaining DNA sequences is dedicated to the production of noncoding RNAs (ncRNA), which play important regulatory functions and constitute up to 96% of the cell RNA [2]. Among the different types of ncRNA are microRNAs (miRNAs). These are responsible for the regulation of human transcriptome and, as a consequence, involved in many physiological and pathophysiological processes. The biological importance of miRNAs, initially identified in cell differentiation and cancerogenesis, has sparked a scientific quest to also understand their role in cardiovascular disease (CVD). In this review, we will briefly describe the biogenesis of miRNAs, together with their role in pathophysiology and their potential applicability as new biomarkers for CVD.

MICRORNAS: DEFINITION, BIOGENESIS, AREAS OF RESEARCH

MicroRNAs (miRNAs, miRs) are short, evolutionary conserved sequences, numbering between 17 and 25 nucleotides in length. MiRNAs bind to the 3′ untranslated regions of messenger RNAs (mRNAs), and induce degradation or inhibition of protein translation. Through their ability to regulate gene expression at the posttranscriptional level, they are able to modify a wide range of biological processes such as cell proliferation, differentiation, apoptosis, and stress response [3]. Their power as regulators is demonstrated by the fact that up to 30–50% of genes are controlled by miRNAs [4]. The most recent version of the central miRNAs database (v21.0), available at the website miRBase.org, contains 35,828 mature miRNA products, in 223 species. To date, there have been 1,881 precursors and 2,588 mature miRNAs annotated and described in humans [5].

MiRNAs are initially transcribed from nuclear DNA to long primary transcripts (pri-miRs) by the RNA-polymerase II, and the majority of them are subsequently spliced by the Drosha RNase III-cofactors complex. Transported out of the nucleus by exportin-5, pre-miRs are cleaved by Dicer RNA-processing ribonuclease III into small double stranded miR-miR* duplexes (Fig. 1). The mature (functional) miR strand is loaded into the RNA-induced silencing complex, where it can guide the mRNA silencing by either degradation or translational inhibition, whereas the complementary passenger strand miR is degraded or incorporated into small vesicles called exosomes and released from the cell. Both pre-miRNA and mature miRNA present in cytoplasm can be released from the cell in microvesicles. MiRNAs circulate in the blood in association with RNA-binding proteins argonaute2 (Ago2) or high-density lipoproteins. The transport out of the cell may be conducted passively in apoptotic bodies, or actively through the channel proteins [6]. Released miRNAs can then act as intercellular communicators and influence gene expression in the target cells [7].

The function of miRNA has been most thoroughly investigated to date in the field of oncology. By regulating differentiation in both normal and cancer cells, miRNAs can act as tumour oncogenes or tumour suppressors [8]. Their role in the development of several types of human neoplasms has been identified [9]. For instance, polymorphism in miR-146a is a predisposing factor for thyroid carcinogenesis [10, 11]. As a consequence, miRNAs are emerging as new prognostic

Address for correspondence:
Dominika Klimczak, MD, Division of Heart Failure and Cardiac Rehabilitation, Department of Cardiology, Hypertension and Internal Medicine, Second Faculty of Medicine, Medical University of Warsaw, ul. Kondratowicza 8, 03–242 Warszawa, Poland, e-mail: dominika.klimczak@yahoo.com

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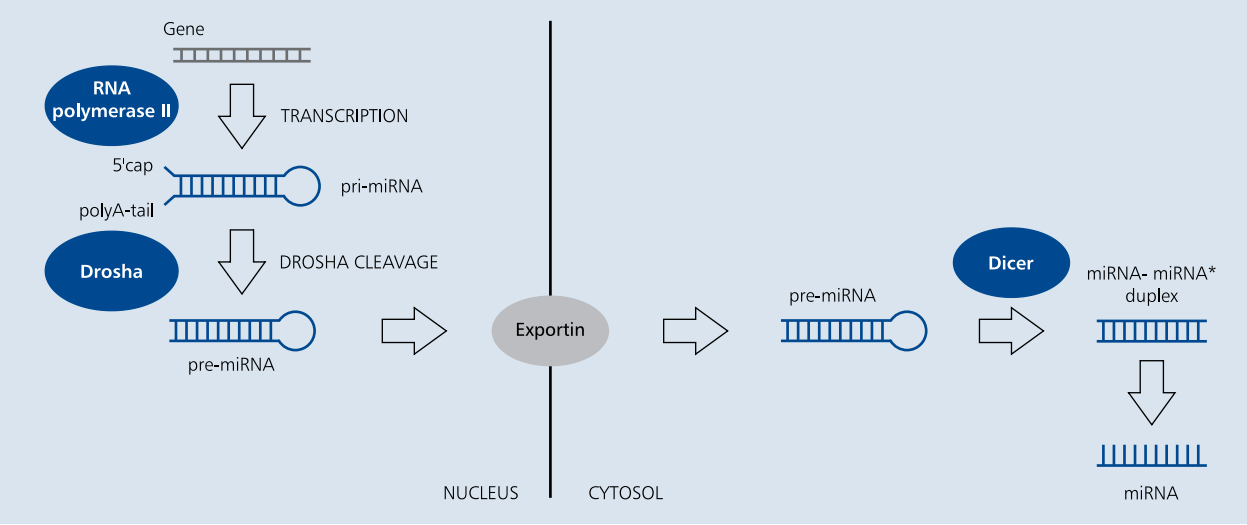

Figure 1. MicroRNA synthesis

and diagnostic tools in oncology that, for instance, could potentially be of use in tracing the origin of metastases [12].

THE ROLE OF MIRNAS IN THE CARDIOVASCULAR SYSTEM

Experience from the field of oncology has been transferred to the area of CVD. We will briefly describe a few examples of miRNA deregulation in cardiovascular conditions.

Vessel wall degeneration, platelet function and atherosclerosis

MiRNAs play an important role in the progression of atherosclerosis and neointimal hyperplasia through their involvement in crosstalk between endothelial and vascular smooth muscle cells [13]. They regulate several pathways involved in cell proliferation, migration and apoptosis [14]. MiRNAs play a role in the degenerative processes of the vessel wall. For instance, miR-29 predisposes to aneurysm formation [15].

The function of platelets, central to coronary artery disease (CAD) development, is also subjected to regulation by miRNAs. According to the most recent data, human platelets express 284 miRNAs. They influence the expression of platelet proteins, such as receptor P2Y(12) [16]. MiRNA profiles are associated with platelet reactivity, may serve as its predictors, and can act as biomarkers of platelet activation [17].

In a rat model of acute myocardial infarction (AMI), specific miRNAs i.e. miR-1, miR-133a, and miR-499 in plasma were increased at 1–3 h, with a peak at 3–12 h and a decrease at 12–24 h after coronary artery ligation, enabling the investigators to hypothesise that they may serve as a marker of myocardial necrosis [18]. Interestingly, miRNAs also play a regulatory role in the response to hypoxia by the cadherin/Wnt and HIF-1 (hypoxia inducible factor) pathways [19].

Cardiac hypertrophy and heart failure

MiRNAs play a role in both the physiological and the pathogenic processes in cardiac hypertrophy. Altered miRNA expression is associated with modulation of cardiac transcriptome and activation of the foetal gene programme [20]. The function and growth of cardiomyocytes is regulated by different signalling pathways including thyroid hormone, and IGF1/Akt, calcineurin/NFAT, and TGF-beta/Smad which are associated with the following miRNAs: miR-208a, miR-499, miR-1, miR-27b, miR-133, miR-23a, and miR-199b. However, cardiac hypertrophy is the result of processes that involve not only cardiomyocytes but also non-cardiomyocyte cell types such as fibroblasts, smooth muscle and endothelial cells. The specific profile of miRNAs in these cells is associated with interstitial fibrosis, angiogenesis and inflammation [20]. For instance, cardiac fibroblasts produce and secrete exosomes with passenger strands miRNA-21-3p which induce a significant increase in cardiomyocyte cell size *in vitro* [21].

Cardiac hypertrophy is also the consequence of alterations in the immunological and autonomous nervous system. According to recent data, cardiac hypertrophy results from the action of proinflammatory cytokines such as TNF-alpha, IL-1, IL-6 and INF-gamma which interact with miR-146. Recent studies have shown that the modulation of the beta-adrenergic system is associated with altered expression of miR-133b, miR-92, miR-100 and miR-195 and, as a consequence, alterations in the gene expression profile (Fig. 2) [22].

MiRNAs as potential biomarkers for cardiovascular disease

MiRNAs, first identified in the blood in 2008, can be found as components of serum, plasma and peripheral blood mononuclear cells (PBMCs) [23]. They are resistant to RNase degradation and remain stable in stored samples [24]. There-

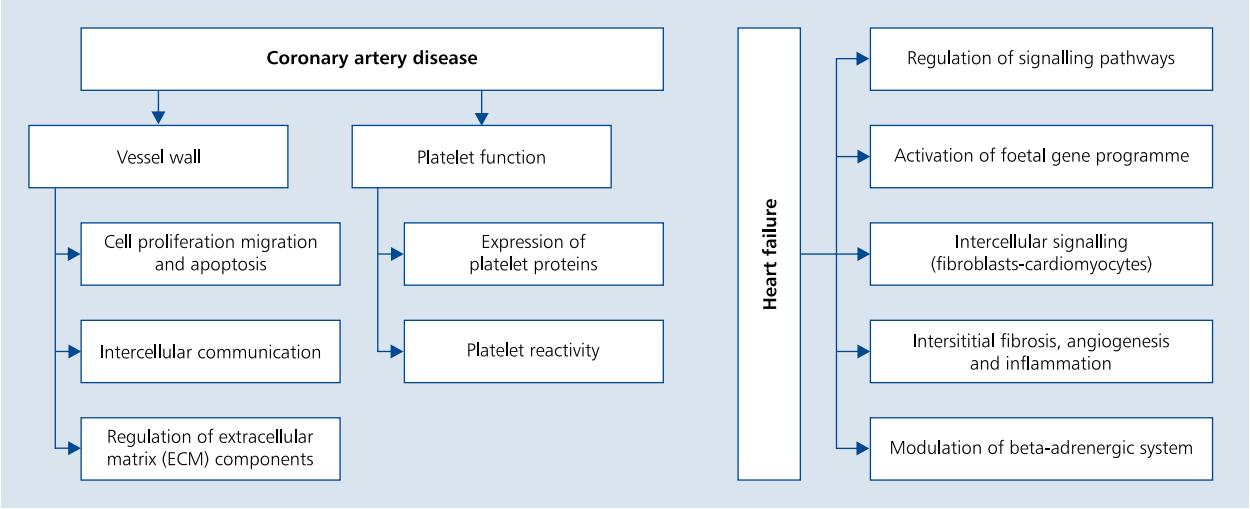

Figure 2. Involvement of miRNAs in cardiovascular pathophysiology

fore it has been hypothesised that miRNAs may be useful as biological markers. The number of miRNAs evaluated so far as potential biomarkers amounts to 416, of which 192 were specific for a disease state. In the field of CVD, 29 studies have reported 87 miRNA biomarkers [23]. The role of miRNA has been investigated most extensively in three cardiovascular conditions: AMI, stable CAD, and heart failure (HF).

Acute myocardial infarction

Of numerous miRNAs evaluated in recent studies concerning their potential role as biomarkers in AMI, three of them (miR-1, miR-133a, and miR-499-5p) were consistently elevated in four studies [18, 25–27].

The levels of these miRNAs, as well as miRNA-133b, were increased in a study evaluating 259 miRNAs expressed in the systemic circulation of AMI patients. Interestingly, miR-1, -133a, and -133b achieved their peak before cardiac troponin I (cTnI). MiR-1, miR-133a, and miR-133b are highly expressed not only in the heart but also in skeletal muscle. Therefore the investigators additionally verified the effect of skeletal muscle ischaemia on miRNA concentration. In the murine model of hind-limb ischaemia used in the study, miR-1, miR-133a, and miR-133b did not increase in ischaemic conditions after femoral artery dissection [25].

The diagnostic value of miR-1, miR-133a, and miR-499-5p, together with miR-208b, was compared to that of troponin T (cTnT). Although the levels of these miRNAs were significantly higher in AMI patients than in healthy volunteers, sensitive and specific for AMI and decreased close to the baseline levels at the time of hospital discharge, receiver operating characteristic curve (ROC) analyses showed that none of them was superior to cTnT for the diagnosis of AMI [26]. However, these results do not enable us to conclusively establish these miRNAs as less sensitive indicators of myocardial necrosis than cardiac troponins because of the following reasons.

Firstly, the samples were collected from the patients within 12 h of the onset of symptoms, which is a quite wide range of time and decreases the reliability of this measurement. Secondly, the sample size was quite small as it amounted to 67 AMI patients.

A similar set of miRNAs (miR-1, miR-133a, miR-208a and miR-499) was evaluated in a study by Wang et al. [18] where miR-208a presented 100% specificity and 90.9% sensitivity, while parallel cTnI was detected only in 85% of patients within 4 h of symptom onset. The results of ROC analysis imply that among these four miRNAs, plasma miR-208a might have the highest sensitivity in an early setting of AMI, as miR-208a presented the highest area under the curve (AUC) of 0.965. Interestingly, plasma level of miR-208a was not detectable in patients with non-CVD including acute kidney injury, chronic renal failure, stroke and trauma using the real-time polymerase chain reaction (PCR) analysis, which makes it better than cTnI in the diagnosis of AMI. The observation that the level of miR-208a became detectable within 1–4 h of chest pain when the cTnI level was still detected below the cut-off value, may be explained by the fact that cTnI is mainly bound to the myofibrils and only 2.8–4.1% of cTnI is cytosolic, whereas miRNAs are bound to protein complex which is predominantly cytosolic. Therefore, cTnI and miRNAs may differ in their release kinetics and miRNAs may be released earlier from the cell in the condition of myocardial injury [18].

In a study on a large sample of 510 myocardial infarction (MI) patients referred for primary mechanical reperfusion, Devaux et al. [27] compared the diagnostic accuracy of miRNAs and hs-cTnT. Overall, miR-208b provided a lower diagnostic accuracy than miR-499 and hs-cTnT with an AUC value of 0.90, whereas miR-499 and hs-cTnT were both able

to accurately discriminate MI from controls with comparable diagnostic sensitivity and specificity (AUC = 0.97 in both cases). The lower accuracy of miR-208b may result from the fact that, in contrast to miR-208a, miR-208b is not cardiac specific and its expression is higher in the skeletal muscle compared to the myocardium [28].

Olivieri et al. [29] investigated a large cohort of 92 elderly non-ST elevation MI (NSTEMI) patients (82.6 ± 6.9 years old) and pointed out that the diagnostic accuracy of miR-499-5p was higher than conventional and hs-cTnT in differentiating NSTEMI vs. acute congestive HF (CHF) patients with modest cTnT elevation at presentation (miR-499-5p AUC = 0.86 vs. cTnT AUC = 0.68 and vs. hs-cTnT AUC = 0.70).

Although the results of the studies mentioned are not fully concordant, two out of these four miRNAs, i.e. miR-208a and miR-499, the so-called MyomiRs (as they are encoded by myosin genes and control cardiac remodelling, muscle myosin content and myofibre identity), appear to be the most accurate candidates for AMI biomarkers [30]. ROC analysis reveals that both miRNAs may be superior to cardiac troponin in the detection of necrosis, whereas the utility of miR-1 and miR-133 still needs to be determined. The increase of miR-1 and miR-133 plasma concentration may not be the result of release from necrotic cells, but may rather be a manifestation of repair mechanisms as these miRNAs are involved in the regulation of myocyte development and electrical properties [26].

Acute coronary syndromes

The promising results of the studies encourage verification of the next issue: whether miRNAs can discriminate between AMI and unstable angina (UA). The levels of six miRNAs previously measured in the studies on AMI patients (MiR-1, miR-133a, miR-133b, miR-208a, miR-208b, and miR-499) were evaluated in a large group of 444 acute coronary syndrome (ACS) patients [31]. Although AMI patients presented higher levels of miR-1, miR-133a, and miR-208b compared to UA patients, all six investigated microRNAs showed a large overlap between these groups of patients.

According to recent research, among the possible factors influencing miRNA levels are also immunological processes. The results of the study by Guo et al. [32] showed that the expression of miR-146a in PBMCs was significantly increased in patients with ACS and led to a significant upregulation of the T helper 1 (Th1) cell function. Proinflammatory phenomena were significantly attenuated by the inhibition of miR-146a. Therefore miR-146a emerges as a new potential therapeutic target in ACS patients [32].

Another study evaluated the role of pro-inflammatory miRNAs in the development of atherosclerosis: miR-155 expression was downregulated, whereas mi-21 and miR-146a were upregulated in PBMCs of patients with UA and AMI. miR-155 may be downregulated as a feedback mechanism that controls the overactivation of immune cells [33].

Stable coronary artery disease

We have previously discussed the role of miRNAs in the development of atherosclerosis at the molecular level. According to the study by Hoekstra [34], based upon a relatively high expression level of a cluster of three miRNAs including miR-134, miR-198, and miR-370, UA patients could be distinguished from stable patients, suggesting that the miRNA signatures could be used to identify patients at risk for ACS.

Plasma levels of miRNAs have been investigated in patients with hyperlipidaemia, also a major risk factor for CAD. According to the data from *in vivo* and *in vitro* studies, miR-122, miR-370, and miR-33a/b play crucial roles in lipid metabolism influencing fatty acid synthesis and oxidation, affecting plasma total cholesterol and triglyceride plasma concentrations. A study revealed that plasma levels of miR-122 and miR-370 were higher in patients with hyperlipidaemia than in controls. Patients on statin therapy presented significantly lower expression of miR-122 and miR-370 compared to statin-free counterparts. Plasma levels of miR-122 and miR-370 were significantly associated with the presence (but not the severity) of CAD, even after adjusting for age, gender, body mass index, smoking, hypertension, diabetes, and blood lipid profiles. However, plasma concentrations of miR-33a and miR-33b were below the detection limit in both the hyperlipidaemia and control groups [35].

Heart failure

The study by Tijsen et al. [36] aimed to verify whether miRNAs allow the distinction of clinically overt HF from healthy controls and non-HF forms of dyspnoea. Seven miRNAs (miR-423-5p, miR-18b*, miR-129–5p, miR-1254, miR-675, HS_202.1 and miR-622) were significantly elevated compared to healthy controls. However, all analysed miRNAs (apart from miR-423-5p) were also slightly increased in non-HF dyspnoea cases. miR-423-5p was a diagnostic predictor of HF with an AUC of 0.91 with a high predictive power when comparing HF and non-HF cases (AUC = 0.83). Although the six miRNAs are not so potent predictors of HF, based on the results of this study we cannot conclusively exclude the five miRNAs from the group of biomarker candidates, as even NT-pro B-type natriuretic peptide (BNP), a well-validated biomarker of HF, can be slightly increased in a case of pulmonary disease leading to right ventricular overload. Interestingly, miR-423-5p was higher in atherosclerotic forms of HF. Unfortunately, the origin of miR-423-5p in this group of patients in uncertain. Its upregulation may be the result of foetal cardiac reprogramming in a failing human heart, a process which involves many miRNAs [36]. An array study of cardiac tissue revealed that 86.6% of induced miRNAs and 83.7% of repressed miRNAs were regulated in the same direction in foetal and failing heart tissue compared to a healthy adult control left ventricle [37].

In a study of patients with ischaemic heart disease, downregulation of miR-126 was associated with CHF. The

investigators observed upregulation of miR-126, with an improvement of the clinical condition of patients from New York Heart Association (NYHA) class IV to class III. Its level negatively correlated with age and logBNP [38]. MiR-126 is highly enriched in endothelial cells. It is possible that a perfusion defect or cellular ageing may reduce the metabolic activity and endothelial cells renewal, leading to a reduction in the release of miRNAs. The alterations in miRNA-126 level may also be not directly linked to HF but rather reflect the presence of additional conditions known to be associated with miRNA-126 downregulation such as CAD and diabetes mellitus type 2, which are more likely to occur in patients with severe CHF with NYHA classes III and IV. Another hypothesis is that endothelial activation may induce the degradation of circulating miRNAs. Therefore, the diagnostic accuracy of miR-126 in CHF needs to be determined in larger cohorts [39].

Another miRNA investigated as a potential biomarker of CHF was hypoxia associated miR-210 (investigated in stroke as well). The measurement of miR-210 in plasma revealed an increase [40]. In the study, the tissue expression of miR-210 in an HF rat model was increased in skeletal muscles and mononuclear cells and remained unchanged in the heart and the kidneys (probably due to the fact that blood perfusion of these organs may be preferentially preserved). Therefore, plasma miR-210 levels may reflect a mismatch between the pump function of the heart and oxygen demand in the peripheral tissues, and be a new biomarker for chronic HF in addition to plasma BNP concentrations [40].

Goren et al. [41] carried out a study wherein the levels of 186 miRNAs were measured in the sera of stable chronic systolic HF patients. The levels of four miRNAs i.e. miR-423-5p, miR 320a, miR-22, and miR-92b were the most significantly increased in sera of HF and presented an association with some of the clinical and prognostic parameters. The levels of the four miRNAs were combined into a single score which could distinguish the HF group from the control group as demonstrated by the ROC curve with an AUC of 0.90. A significant association was observed between the miRNA-score and several validated prognostic parameters including: elevated serum BNP levels, a wide QRS, and dilatation of the left ventricle and left atrium. A summary of miRNAs evaluated as biomarkers is presented in Table 1.

Table 1. MiRNAs evaluated as potential biomarkers

Acute myocardial infarction	miR-1, miR-133a, miR-133b, miR-208a, miR-208b, miR-499-5p
Unstable angina	miR-134, miR-198, miR-370
Heart failure	423-5p, miR-18b*, miR-129-5p, miR-1254, miR-675, HS_202.1, miR-622, miR-126, miR-210, miR 320a, miR-22, miR-92b

CHALLENGES AND FUTURE PERSPECTIVES

MicroRNAs appear to be an attractive class of biomarkers, thanks to their advantages such as: stability in extracellular milieu and body fluids including blood, urine and saliva, evolutionarily conserved sequences, highly sensitive and specific detection method used (real-time PCR), non-invasive measurability, high dynamics of change in the course of the disease, long half-life within the sample and, in some cases, tissue specificity [42, 43].

However, there are some challenges to address before we apply miRNAs in clinical practice. Firstly, the changes in expression level of many miRNA species are often associated with multiple conditions (for instance miR-21 in heart muscle hypertrophy and breast cancer) [42, 44]. Besides, there are some problematic methodological aspects that include: lack of standard protocols, interference of anticoagulants in PCR based profiling, low recovery of miRNAs and difficulties with endogenous controls [45].

Future perspectives

MiRNAs have promising potential for their clinical application as biomarkers. However, many questions are still unresolved. It requires further investigation as to what factors govern the release mechanisms of miRNAs from a cell and what are the targets of specific miRNAs in cell-to-cell communication. The changes in the expression level of many miRNAs have unknown origin and significance.

Another field which requires further exploration is the therapeutic use of miRNAs. The therapy may be based on two approaches: restoring the function of miRNA (using synthetic double-stranded miRNAs as an example), or inhibiting miRNA function by the use of chemically modified antimirR oligonucleotides. Several antimiRs of potential application in CVD are already in the preclinical trial phase: miR-33 in atherosclerosis, miR-208 in HF and cardiometabolic disease, miR-15/195 in post MI-remodelling, miR-145 in vascular disease, and miR-92 in peripheral artery disease. So far the only clinical phase II study of miRNA completed, investigating antimir-122 (Miravirsen), reports its safety and good tolerance as well as prolonged antiviral activity in the treatment of chronically HCV-infected patients [46].

CONCLUSIONS

MiRNAs are a new generation of potential biomarkers. The list of miRNAs identified in human blood is growing all the time. The lack of large cohort studies is the main obstacle to transferring miRNAs analyses to clinical practice. There have been some good candidates identified for use in specific conditions, such as miR-208a and miR-499 in AMI or miR-423-5p in HF, which require validation on a larger study sample. MiRNAs may constitute additional prognostic value to biomarkers of CVD available at the moment, as well as serving as independent prognosticators and disease monitoring tools. Additionally, although much still remains to be learned

about miRNA biology, chemistry and delivery systems, they provide an opportunity of advance towards novel therapeutics. The application of this class of molecule is a step towards the development of personalised medicine.

Conflict of interest: *none declared*

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