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SPOTLIGHT REVIEW

Profiling of circulating microRNAs: from single biomarkers to re-wired networks

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Abstract The recent discovery that microRNAs (miRNAs) are present in the circulation sparked interest in their use as potential biomarkers. In this review, we will summarize the latest findings on circulating miRNAs and cardiovascular disease but also discuss analytical challenges. While research on circulating miRNAs is still in its infancy, high analytical standards in statistics and study design are a prerequisite to obtain robust data and avoid repeating the mistakes of the early genetic association studies. Otherwise, studies tend to get published because of their novelty despite low numbers, poorly matched cases and controls and no multivariate adjustment for conventional risk factors. Research on circulating miRNAs can only progress by bringing more statistical rigour to bear in this field and by evaluating changes of individual miRNAs in the context of the overall miRNA network. Such miRNA signatures may have better diagnostic and prognostic value.

Keywords MicroRNA • Cardiovascular disease • Diabetes • Atherosclerosis • Systems biology

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1. Introduction

microRNAs (miRNAs) are small non-coding regulatory molecules of ∼22nt length that modify gene expression at the post-transcriptional level by binding to the 3′ untranslated region (UTRs) of their target messenger RNAs (mRNAs). Although the exact mechanism of action is still unclear, it is accepted that at least in mammals miRNAs bind through imperfect complementarity to their target genes. Nucleotides 2–8, the so-called 'seed region', are thought to be essential for this interaction, while the 3′ binding—although not necessary—may compensate for weak seed binding.[1] Several mechanisms of action have been proposed for miRNA-mediated gene repression. Protein synthesis may be suppressed by inhibition of translational initiation, mRNA degradation due to deadenylation or in rare cases mRNA cleavage.[2–4] Evolutionary conserved, miRNAs constitute a layer of epigenetic regulation that provides an additional control of intricate processes such as cell growth, differentiation, stress response, and tissue remodelling. They may act as buffers to absorb perturbations and safeguard the robustness of biological systems. There is accumulating evidence for a role of miRNAs as key regulators in the cardiovascular system: displaying distinct tissue expression profiles, miRNAs orchestrate cardiac development, vessel wall homeostasis, response to vascular injury, angiogenesis, and tissue repair.[5,6] Circulating miRNAs may not just represent the 'spill-over' of the cellular miRNA content,[7] but may also contribute to inter-cellular signalling and offer new insights into pathological mechanisms.[8–10] In this review, we will focus on the potential of circulating miRNAs as biomarkers of cardiovascular disease and discuss their potential as well as their analytical challenges.

2. Stability and compartmentalization of circulating microRNAs

In a seminal paper, Mitchell *et al.*[11] demonstrated the presence of endogenous miRNAs circulating in human plasma. The authors incubated plasma samples at room temperature for 24 h, performed repeated freeze-thaw cycles and assessed the expression of miR-15b, miR-16, and miR-24 by quantitative PCR (qPCR). Unlike

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mRNAs, circulating miRNAs displayed remarkable stability[12] and resistance to degradation from endogenous RNAse activity.[13,14] In contrast, rapid degradation was observed within minutes when synthetic miRNAs (corresponding to *Caenorhabditis elegans* miRNAs cel-miR-39, cel-miR-54, cel-miR-238) were spiked into human plasma. Once the denaturing solution inactivated RNase activity, the exogenous miRNAs escaped degradation. Thus, naked miRNAs are susceptible to rapid degradation in plasma, whereas circulating miRNAs are protected and resistant to RNase activity. The following explanations have been proposed: Circulating miRNAs can reside in microvesicles [exosomes, microparticles (MPs), and apoptotic bodies (ABs)], which account for shedding of miRNAs into the circulation and offer protection from RNase activity. Other studies demonstrated that after isolation of microvesicles and subsequent high-speed centrifugation of both cell culture medium[15,16] and plasma[10,17,18] miRNAs were still detectable in the fraction devoid of microvesicles, suggesting the existence of non-vesicle associated miRNA-protein/lipoprotein complexes in extracellular fluids (*Figure 1*).

2.1 Intravesicular transport

2.1.1 *miRNAs in exosomes*

Exosomes are small vesicles (50–90 nm) of endocytic origin.[19,20] They are stored as intraluminal vesicles within the multivesicular bodies of the late endosome and released by fusion with the cell membrane. In response to cell stimulation, budding of endosomes occurs, a process dependent on calcium influx, calpain and cytoskeleton reorganization[21] and sphingomyelinase 2 activity (nSMase2), the rate-limiting enzyme of ceramide biosynthesis, which controls the release of exosomes.[22] Exosomes contain substantial amounts of RNA,[23,24] including both mRNA and miRNAs.[15,24–29] A total of 121 miRNAs were identified in exosomes from mast cells and the expression of certain miRNAs was higher in microvesicles than in the parent cells.[29] In contrast, selective retention of specific miRNAs that are not released into the extracellular milieu was reported in cancer, implying that miRNAs can be selectively packaged.[26]

2.1.2 *miRNAs in microparticles*

Microparticles are larger than exosomes (>100 nm diameter), form through plasma membrane budding and also contain miRNAs.[30,31] Blebbing of MPs from the cell membrane requires increased intracellular calcium, changes in membrane lipid asymmetry, and cytoskeleton protein reorganization. This leads to exposure of phosphatidylserine on the outer leaflet as a consequence of the calcium-dependent activation of scramblase and floppase/ABC1 and the inhibition of translocase/flippase activities. Phosphatidylserine on the MP surface promotes coagulation[32] and serves as a recognition signal for clearance of senescent and apoptotic cells.[33–36] Although MPs were initially considered 'cell debris', experimental evidence suggests that MPs influence diverse biological functions.[37,38] Microparticles can transfer chemokines, adhesion molecules, receptors, and bioactive lipids.[39] Depending on the stimulus, circulating MPs differ in their number, composition, and cell origin.[40] In general, their numbers tend to increase in cardiovascular disease.[41–43] The presence of a miRNA pool has now been reported for MPs from a variety of cell types including mast cells, platelets, endothelial cells (ECs), and monocytes. Importantly, different stimuli alter the release of miRNAs in MPs, suggesting that similar to exosomes the miRNA export in MPs is an actively regulated process.[10]

2.1.3 *miRNAs in apoptotic bodies*

Larger in size than MPs, ABs are generated in response to apoptotic stimuli.[44] ABs have been implicated in tissue repair and angiogenesis while their engulfment by phagocytes triggers the secretion of cytokines or growth factors.[45] Microarray analysis of the miRNA content of ABs derived from ECs highlighted the presence of a panel of miRNAs that reflected its cellular origin and revealed several enriched miRNAs compared with the cellular miRNA content.[9] miR-126 was the most abundant miRNA in endothelial ABs. miR-126 is a key regulator of vascular endothelial growth factor (VEGF) and fibroblast growth factor signalling in ECs through inhibition of two negative regulators of the VEGF signalling pathway.[46,47] Deletion of miR-126 in mice resulted in partial embryonic lethality due to loss of vascular integrity and haemorrhages. A novel function of miR-126 residing in ABs was reported in atherosclerosis: endothelial cell-derived ABs mediated the induction of CXCL12 in a miR-126-dependent manner resulting in the mobilization and incorporation of stem cell antigen-1 positive progenitor cells to atherosclerotic plaques and a decrease in diet-induced atherosclerosis as well as collar-induced plaque formation.[9]

2.2 Extravesicular transport

2.2.1 *miRNAs in lipoprotein complexes*

In a recent study, it was proposed that high-density lipoprotein (HDL) besides its classical role as a delivery vehicle for excess cellular cholesterol also functions as a transporter of endogenous miRNAs. In resemblance to artificial gene delivery vehicles, native HDL is associated with miRNAs, acting as a carrier or depot for circulating miRNAs in plasma and facilitating their transport and delivery to recipient cells.[18] Highly purified HDL negative for exosomal marker proteins was shown to be rich in small RNA molecules, 15–30 nucleotides in length, but devoid of long mRNAs. Total RNA extracted from HDL and exosomes originating from plasma of healthy individuals revealed that their miRNA profile is distinct. A specific miRNA signature of HDL–miRNA complexes was identified in patients with familial hypercholesterolaemia, including hsa-miR-22, hsa-miR-105, and hsa-miR-106a.

2.2.2 *miRNAs in protein complexes*

Argonaute2 (Ago2) is part of the miRNA silencing complex. Ago2/miRNA complexes were identified in cell culture media[16] and immunoprecipitation of Ago2 from plasma recovered circulating miRNAs that are not associated with microvesicles.[17] These stable complexes were further characterized using size-exclusion chromatography to exclude a contamination with microvesicles: whereas the majority of miRNAs co-purified with Ago2 complexes, certain miRNAs, such as miR-16 and miR-92a but not let-7a, associated predominantly with microvesicles. A mechanism involving cell type-specific release in either microvesicle or Ago2 complexes was proposed but requires further elucidation. Notably, Ago2 is not the only miRNA-binding protein released in the cell culture supernatant. A total of 12 RNA-binding proteins were identified by mass spectrometry in the conditioned medium of human fibroblasts following 2 h of serum starvation,[15] including nucleophosmin (NPM1). Direct binding of NPM1 protected miRNAs from degradation. The role of the other RNA-binding proteins is currently unclear, but they had apparently no protective effect against the degradation of synthetic miR-122, at least *in vitro*.

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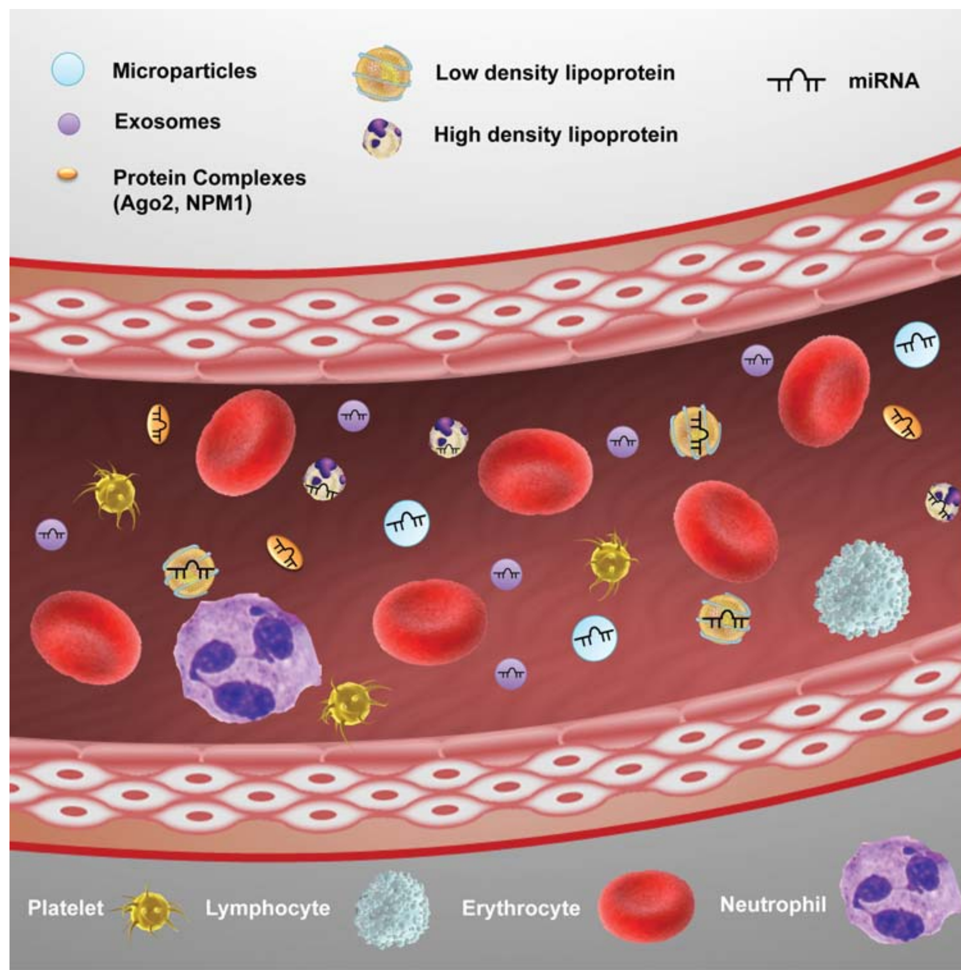

Figure 1 Compartmentalization of circulating miRNAs. Circulating miRNAs are contained within vesicles (exosomes, microparticles, apoptotic bodies), in protein complexes (Ago2, NPM1) and in lipoprotein complexes (HDL, LDL complexes). Although various tissues contribute to the circulating miRNA pool, most miRNAs are probably derived from blood cells. In response to injury, such as AMI, cardiac-specific miRNAs, which are otherwise undetectable are released into the circulation.

3. Circulating miRNAs as a novel mechanism for intercellular communication?

Besides their potential as biomarkers, circulating miRNAs may act as a delivery system and could play a previously unrecognized role in modulating cell function. Interaction of exosomes with recipient cells is thought to arise through receptor-ligand interactions,[48] although fusion to the plasma membrane of target cells or endocytosis-like internalization of exosomes has also been observed. In an *in vivo* model that enabled close monitoring of miR-16 activity, nude mice were implanted with cells engineered to express a reporter for the 3′UTR of B-cell lymphoma 2, a validated target of miR-16. Subsequent intratumour injection of exosomes derived from HEK293 cells overexpressing miR-16 led to suppression of luciferase activity. Control exosomes had no effect on bioluminescence. These data indicate that exosomal miR-16 delivered its inhibitory ability on its target gene to the recipient cells *in vivo*.[49] Similarly, secreted miR-150 from monocytic cells was shown to enhance endothelial cell migration. miR-150 is enriched in leucocytes and lymphocytes, and its presence in microvesicles is increased following stimulation of cells with proinflammatory stimuli. miR-150 is known to target c-Myb, a transcription factor related to cell proliferation, lineage commitment, and migration.[50] Delivery of microvesicles from monocytic cells overexpressing miR-150 down-regulated c-Myb in recipient endothelial cells and enhanced their migration. Moreover, increased circulating levels of miR-150 were detected in patients with severe atherosclerosis. Microvesicles isolated from these patients induced a similar response in endothelial cells supporting the idea of a miRNA crosstalk between monocytes and endothelial cells in disease.[10] Direct delivery of miRNAs to recipient cells can also occur by HDL through a ceramide signalling pathway dependent manner. The miRNAs within HDL altered the cellular miRNA pool and were functional as shown by a loss of corresponding miRNA targets. Thus, besides the well-established role of HDL for reverse cholesterol transport and as a prognostic marker for cardiovascular disease, its miRNA content might have biological relevance.[18] Similarly, Ago2 may not just be a carrier of circulating miRNAs, but the miRNA–protein complexes could have a functional role in silencing gene regulation in recipient cells.[17] Although microvesicles and HDL–miRNA complexes were capable of affecting the expression of target genes in recipient cells

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and inducing cellular responses, conclusive data on the functional role of endogenous extracellular miRNAs *in vivo* are still missing.

4. Circulating miRNAs as biomarkers in cardiovascular diseases

Expression signatures of circulating miRNAs are emerging as novel biomarkers for cardiovascular disease. The miRNA content in the circulation is likely to reflect the activation state of circulating cells and may provide an integrated read-out of cell activation and tissue injury in response to cardiovascular risk factors and disease.

4.1 Acute myocardial infarction

4.1.1 *miR-1*

The putative diagnostic and prognostic value of cardiac miRNAs has been studied in a rat model of acute myocardial infarction (AMI) induced by coronary ligation. Levels of circulating miR-1 showed a strong positive correlation with myocardial infarct size, peaking at 6 h and returning to baseline 3 days post-AMI. Circulating miR-1 was also increased in patients with AMI[51] and positively correlated to serum creatine kinase-MB (CK-MB).[52] These findings were corroborated in another study, which showed a correlation between plasma levels of miR-1 and the QRS complex.[53]

4.1.2 *miR-208*

While miR-1 is highly expressed in heart and skeletal muscle, miR-208 expression is supposed to be restricted to the heart. Its circulating levels were elevated following isoprotenol-induced myocardial injury in rats.[54] This response was not observed after renal injury, suggesting that circulating miR-208 is specific for cardiac injury. As expected, there was a good association between miR-208 and cardiac troponin I (cTnI), an established blood marker of AMI[54] supporting the notion that circulating miRNAs can serve as biomarkers of tissue injury.[14] In humans, microarray screening identified miR-208a as being exclusively expressed in the heart. Indeed, miR-208a was undetectable in healthy individuals and not affected by injury in other tissues such as acute kidney injury, chronic renal failure, stroke, and trauma. Instead, miR-208a displayed both high sensitivity and specificity for AMI.[55] A rise in miR-208a was evident within 1–4 h after the onset of chest pain when cTnI was not yet affected, indicating that miRNAs may leak into the bloodstream at an earlier stage of myocardial injury (the biological peak of troponins is ∼14–18 h after timed AMI).

4.1.3 *miR-499*

In a separate study, circulating miRNAs were isolated from patients with AMI, viral myocarditis, diastolic dysfunction, and acute heart failure (HF). Apart from miR-208b, a significant increase in cardiac myocyte-associated miR-499 was observed in AMI. Both miRNAs correlated with circulating cardiac troponin T (cTnT), and were modestly elevated in viral myocarditis. Circulating levels of the leucocyte-expressed miRNAs (miR-146, miR-155, and miR-223) were similar in patients with AMI or viral myocarditis despite elevated white blood cell counts. On the other hand, individuals with acute HF had higher levels of miR-499, whereas levels were unchanged in diastolic dysfunction.[56]

4.1.4 *miR-133*

Microarray analysis confirmed that miR-1, miR-133a, miR-208a, and miR-499 were significantly reduced in the infarcted myocardium, while circulating miR-1 and miR-133a were increased in patients with AMI, suggesting that these miRNAs originate from the injured myocardium. miRNA profiling of plasma samples derived from patients with AMI revealed increased levels of miR-1, miR-133a, miR-133b compared with healthy subjects.[51] Intriguingly, other highly expressed miRNAs in the heart (miR-24, miR-26a, miR-126, miR-30c) were not affected. To delineate the contribution of the heart and the skeletal muscle, two animal models were compared: in a murine model of AMI, miR-1, miR-133a, and miR-133b levels peaked 6–18 h after coronary occlusion. In contrast, levels of circulating miR-1, miR-133a, and miR-133b showed an early decline in a mouse model of hind-limb ischaemia and returned to baseline within a day.[51]

4.2 Acute coronary syndrome

In the largest study on myocardial infarction to date ($n = 444$), 6 miRNAs (miR-1, miR-133a, miR-133b, miR-208a, miR-208b, and miR-499) were compared in patients with non-ST-elevation myocardial infarction (NSTEMI), ST-elevation myocardial infarction (STEMI), and unstable angina.[57] miR-208b was only detectable in NSTEMI and STEMI patients. Individuals with undetectable miR-208b levels had the best prognosis. Mortality increased with rising levels of circulating miR-208b. miR-133a was detectable in all samples, but higher in patients with NSTEMI or STEMI compared with unstable angina. Circulating concentrations of miR-133a were correlated with all-cause mortality at 6 months in a non-linear U-shaped relationship. Patients in the first and fourth quantiles of miR-133a had the worst prognosis, probably due to the inverse association of miR-133a with younger age. However, multiple regression analysis revealed that miR-208b and miR-133a were associated with high-sensitivity troponin T (hsTnT). After adjustment for hsTnT, both miRNAs lost their independent association with patient outcome.

4.3 Stable coronary artery disease

miRNA profiling suggested that cardiac enriched miR-133a and miR-208a were higher in patients with stable coronary artery disease. Additionally, endothelial enriched miR-126 and the cluster miR-17–92 (miR-17, miR-20a, miR-92a) were down-regulated in patients, both in the discovery ($n = 36$) and the validation cohort ($n = 31$). Likewise, the inflammation-associated miR-155 was significantly reduced.[58]

4.4 Heart failure

Tijsen *et al.*[59] assessed the plasma miRNA profile in patients with HF ($n = 50$). Healthy controls and patients with other forms of dyspnoea were used as controls. A total of 16 miRNAs were validated by qPCR but only 10 of the candidates identified by the array were differentially expressed, including miR-423-5p. miR-423-5p emerged as a significant predictor of HF in a multivariate logistic regression model including age and sex. miR-423-5p distinguished HF cases from healthy controls and patients within other forms of dyspnoea not related to HF. Concerns, however, were raised regarding the validity of the statistical analysis and the size of the study population.[60] Clearly, larger clinical studies are needed to determine the clinical value of miR-423-5p as a biomarker of HF.

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4.5 Hypertension

Screening for circulating miRNAs revealed a novel link between human cytomegalovirus and essential hypertension.[61] A panel of 27 differentially expressed miRNAs was identified. Validation of a subset of putative targets confirmed altered expression of miR-296-5p, let-7e, and hcmv-miR-UL112, a human cytomegalovirus-encoded miRNA. Interferon regulatory factor 1 was shown to be a direct target of hcmv-miR-UL112. Seropositivity and high antibody titres to cytomegalovirus were more common in hypertensive individuals. Thus, the miRNA profile led to potential new insights into the pathogenesis of this disease.[61] The role of miRNAs in secondary hypertension has not yet been explored.

4.6 Type II diabetes

We have recently performed the first population-based prospective study on circulating miRNAs ($n = 822$). Interestingly, miR-126 was among the miRNAs most consistently associated with type II diabetes (DM). When circulating levels of miR-126 were determined in the entire Bruneck cohort ($n = 822$, 1995 evaluation), miR-126 was associated with current (1995) and subsequent DM (1995–2005) and inversely correlated with its severity. In logistic regression analyses, miR-126 emerged as a significant predictor of manifest DM [odds ratio (95% CI) for a 1-SD unit decrease in loge-transformed expression level of miR-126, 2.23 (1.69–2.96); $P = 2.28 \times 10^{-8}$] and this association persisted in a multivariable model [odds ratio (95% CI), 1.64 (1.19–2.28); $P = 0.0027$]. Moreover, there was a gradual decrease in circulating levels of miR-126 across categories of normal glucose tolerance ($n = 580$), impaired fasting glucose/impaired glucose tolerance ($n = 162$) and manifest DM ($n = 80$) (*Figure 2*). These results from diabetic patients were corroborated using an animal model of hyperglycaemic obese mice (Lep^{ob}). Endothelial cells constitute a preferred target of DM-induced damage because they lack a regulated control of glucose influx. Primary consequences of glucose overload manifest as endothelial dysfunction, increased vascular permeability and microvascular cell loss by apoptosis. Systemic endothelial dysfunction alters the production of vasoactive substances and reactive oxygen species and modification of the basement membranes is believed to play a decisive role in the vascular complications of DM leading to impaired angiogenesis and collateral vessel development. Indeed, loss of circulating miR-126 emerged as a positive predictor of subclinical and manifest peripheral artery disease in DM. In detail, miR-126 was associated with a low ankle-brachial index (<0.9, $n = 77$) [unadjusted and age-/sex-adjusted odds ratio (95% CI) for a 1-SD unit decrease in loge-transformed expression level of miR-126, 1.95 (1.48–2.57) $P < 0.001$ and 1.39 (1.04–1.86) $P = 0.025$] and with new-onset symptomatic peripheral artery disease (1995–2005, $n = 15$) [unadjusted and age-/sex-adjusted hazard ratio (95% CI) for a 1-SD unit decrease in loge-transformed expression level of miR-126, 2.63 (1.38–5.02) $P = 0.0032$ and 2.15 (1.06–4.38) $P = 0.030$]. These findings suggest that miRNA signatures may have diagnostic and prognostic value in DM,[62] but require validation in independent cohorts. Also, the mechanism for the loss of miR-126 is currently unclear. In principle, changes in circulating miRNAs should be the net effect of altered secretion, degradation, and cellular uptake. In a model of hyperglycaemia, endothelial cells cultured in conditions of high glucose did not show differences in miR-126 expression but exhibited reduced packaging of miR-126 to ABs.[62] Thus, it is tempting to speculate that selective export of miRNAs contributes to the reduced levels of circulating miR-126. However, further studies are required to delineate the cellular origin of miR-126 in the circulation and to clarify whether loss of miR-126 in DM is associated with impaired responsiveness to VEGF contributing to defects in collateral vessel development.[63]

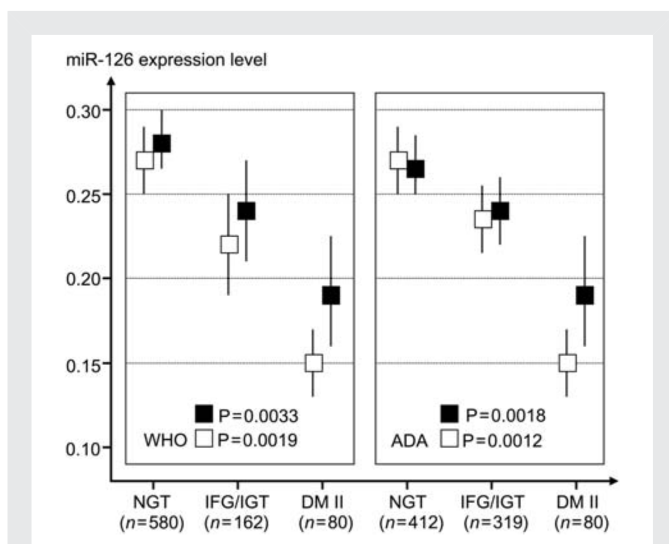

Figure 2 Circulating levels of miR-126 across categories of normal glucose tolerance (NGT), impaired fasting glucose/impaired glucose tolerance (IFG/IGT) and manifest DM. Squares and lines indicate adjusted geometric means and 95% CIs [white squares: values adjusted for age and sex and black squares: values adjusted for age, sex, social status, family history of DM, body mass index, waist-to-hip ratio, smoking status, alcohol consumption (g/day), physical activity (sports index) and high-sensitivity C-reactive protein]. This analysis was performed in the entire study population ($n = 822$). Differences in miR-126 between categories of NGT, IFG/IGT and DM were compared with General Linear Models (GLM) and *P*-values are for trend. World Health Organization (WHO) definition of categories: normal glucose tolerance (fasting glucose < 110 mg/dL and 2 h glucose < 140 mg/dL), impaired fasting glucose/impaired glucose tolerance (110 mg/dL $\leq$ fasting glucose < 126 mg/dL, 140 mg/dL $\leq$ 2 h glucose < 200 mg/dL) and manifest DM. American Diabetes Association (ADA) definition of categories: normal glucose tolerance (fasting glucose < 100 mg/dL and 2 h glucose < 140 mg/dL), impaired fasting glucose/impaired glucose tolerance (100 mg/dL $\leq$ fasting glucose < 126 mg/dL, 140 mg/dL $\leq$ 2 h glucose < 200 mg/dL) and manifest DM (reproduced with permission from Zampetaki *et al.*, Circ Res, 2010[62]).

Adapting a different approach for identifying circulating miRNAs in DM, Kong *et al.*[64] explored the expression of miRNAs involved in insulin biosynthesis and secretion, such as miR-9, miR-29a, miR-30d, miR34a, miR-124a, miR146a, and miR-375. All seven miRNAs were significantly up-regulated in diabetic patients compared with controls. Unlike miR-126, no significant differences were detected between subjects with prediabetes and healthy controls suggesting that a rise in insulin-regulating miRNAs does not precede the development of the disease. Similarly, increased circulating levels of miR-503 were recently reported in diabetic patients undergoing foot amputation for critical limb ischaemia ($n = 11$).[65] While this miRNA may leak into the plasma in advanced stages of disease, i.e. after muscle necrosis, it remains unclear whether miR-503 is detectable in patients with less advanced DM.[66]

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5. Analytical challenges for circulating miRNAs

Statistical analysis of the association between circulating miRNA levels and disease endpoints faces two major challenges. First, circulating miRNAs are highly correlated ($r = 0.6-0.9$) and conventional statistical methods show poor performance when allowing for highly correlated variables with the statistical phenomenon of multicollinearity yielding imprecise, overfitted and potentially biased effect estimates. Second, testing large numbers of miRNAs (~800 are usually covered by miRNA arrays) in studies with only small numbers of cases leads to an increase in the type I error and a high risk of chance findings. Beyond the usual standards in high-quality epidemiological research, additional analytical standards are a prerequisite for circulating miRNA studies.

5.1 Appropriate study design

Studies must be adequately sized and samples for measurement of miRNAs should be drawn prior to disease manifestation (prospective design). Comparisons between hospitalized patients and healthy controls, for example, are likely to be confounded by medication and common cardiovascular risk factors.

5.2 Lack of accepted standards

For any potential clinical application, circulating miRNA measurements have to be performed with high accuracy and precision. miRNA isolation was initially performed using TRIzol reagent and thus the extracted RNA was contaminated by phenol residues. However, recent technical advances led to commercially available kits that have significantly improved the quality of the miRNA preparations from body fluids. Currently, data normalization is the major concern. Any pre-analytical variation, i.e. haemolysis, affects miRNA quantification.[67,68] Variations in extraction efficiency as well technical variations in the qPCR-based quantification of miRNAs require appropriate internal controls. Synthetic exogenous miRNAs derived from *C. elegans* can be spiked in,[58] but do not account for differences in extraction efficiency. Owing to the lack of generally accepted standards, various endogenous miRNAs (miR-17–5p, miR-1249, miR-454) or small non-coding RNAs (RNU6b) were used for normalization purposes.[7,51,54,59,62] Possible alternatives are the Ct average of a miRNA panel or the total protein content of microvesicles.[10,18] Different methods should be employed to make sure that findings are robust irrespective of the way of standardization. These include spiking of synthetic miRNAs, standardization to the mean of all measured miRNAs, and standardization to individual miRNAs that are detectable at similar levels in all study subjects, not associated with disease, and positioned outside the main clusters in the miRNA network.

5.3 Exploration of miRNA co-expression networks

The complexity of higher organisms is regulated through the coordinated control of biological networks, including miRNAs.[69] Computational networks have previously been generated using miRNA array data.[69,70] For the first time, we have applied a similar approach to circulating miRNAs[62] (*Figure 3*). In such networks, nodes represent distinct miRNAs while edges represent computationally derived relationships, such as co-expression, between miRNA pairs. A network-driven analysis offers additional insights compared with studying up- or down-regulation of individual miRNAs alone. First, availability of graph clustering algorithms such as Markov clustering[71] allows identification of miRNA modules that are likely to be involved in the same biological process[69] or delineation of proximal miRNA pairs.[72] Second, integration of differential expression profiling with network topology—contribution of a single miRNA to the stability of the co-expression network[73]—may provide a more reliable alternative to target prioritization and subsequent validation.[70] Finally, representation of biological data as networks of relationships facilitates data integration across multiple levels of biological complexity and may define contribution of specific miRNAs to systems-wide properties of disease.[70] While network biology is a powerful approach for studying relationships between genes, proteins, and diseases,[74] its application in the biomarker field is at an early stage and further analytical and experimental efforts will be needed to substantiate its utility. Network inference algorithms could be particularly useful to characterize circulating miRNA networks, to unravel rewiring of miRNA profiles under pathological conditions and to test for potential associations of miRNA clusters with disease endpoints.

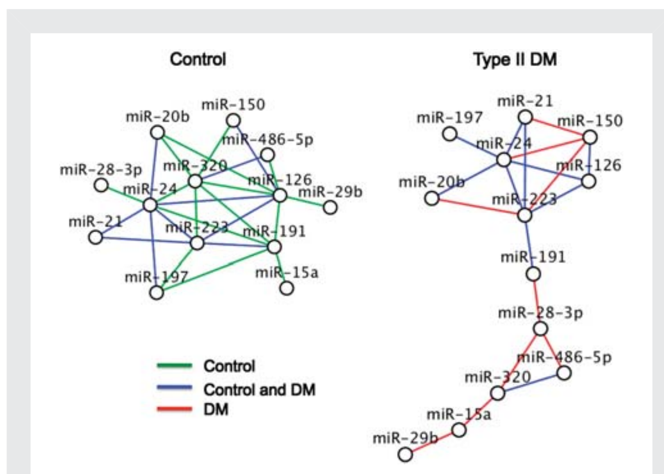

Figure 3 Circulating miRNA networks. Differential network structure between 13 miRNAs in controls and manifest DM. Nodes represent individual miRNAs and edges (links) represent the extent of expression similarity measured using the Context likelihood of relatedness algorithm. For each miRNA topological parameters including node degree, clustering coefficient, and eigenvector centrality can be systematically calculated. Node degree is defined as the total number of edges that are connected to a given miRNA. Clustering coefficient is the degree to which miRNAs tend to cluster together. Eigenvector centrality is a measure of miRNA importance, such that a particular miRNA receives a greater value if it is strongly correlated with other miRNAs that are themselves central to the network. Control (13 nodes, 25 links) and DM (13 nodes, 19 links) networks shared only 10 links. miR-126 occupied a central position within the network and the disease state was characterized by substantial edge rewiring, i.e. for miR-15a (reproduced with permission from Zampetaki *et al.*, Circ Res, 2010[62]).

5.4 L1-penalized Cox regression for high-dimensional and highly correlated data

As mentioned above, common statistical approaches should be complemented by more sophisticated procedures suitable for high-

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dimensional and collinear data. We have successfully applied L1-penalized Cox regression analysis, which was initially developed to generate gene signatures from micro-array data (package 'penalized', freely available).[75]

5.5 Replication in independent cohorts

Rigorous replication of findings in independent population samples and/or experimental animals should be obligatory to minimize the probability of reporting false-positive findings.

5.6 Experimental findings in support of observed associations

Findings gain persuasiveness if corroborated by appropriate experiments highlighting pathophysiological plausibility for the association observed or if findings in patients are replicated in animal models of disease.

6. Conclusion

There are currently no good soluble biomarkers, which could be used to accurately identify subjects who are at risk of developing acute manifestations of cardiovascular disease. Despite extensive studies and development of several risk prediction models, traditional risk factors fail to predict cardiovascular events in a large group of cases (25–50%). Inflammatory markers such as high-sensitivity C-reactive protein are widely used but lack specificity for the vasculature. Advanced imaging techniques are expensive and not suitable for population-wide screening. Besides, atherosclerosis is a diffuse disorder with various local and systemic manifestations. Circulating miRNAs could be attractive biomarkers. They are easily accessible, relatively stable and in some instances tissue specific. On the other hand, the point of care test for cTnI and cTnT can already provide results within minutes of bleeding the patient. Laboratory-based assays such as hsTnT are faster than qPCR-based assays, and the latter will not be of clinical value unless they outperform existing tests for myocardial injury. However, qPCR-based assays are used routinely for the diagnosis of viral infections. Undoubtedly, there is a clinical need for biomarkers of vascular injury that could complement the assessment of traditional risk factors in monitoring vascular health and stratifying patients according to risk and treatment response. It remains to be seen whether miRNAs can fulfil this need and improve risk prediction. At least, circulating miRNAs may offer new insights into the mechanisms and systemic manifestations of cardiovascular disease.

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Conflict of interest: A.Z., I.D., S.K., and M.M. filed a patent application, owned by King's College London, that details claims related to described circulating miRNAs as cardiovascular biomarkers.

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