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Circulating MicroRNAs: Novel Biomarkers and Extracellular Communicators in Cardiovascular Disease?

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Circulating MicroRNAs

Novel Biomarkers and Extracellular Communicators in Cardiovascular Disease?

Esther E. Creemers, Anke J. Tijsen, Yigal M. Pinto

Abstract: **In the past few years, the crucial role of different micro-RNAs (miRNAs) in the cardiovascular system has been widely recognized. Recently, it was discovered that extracellular miRNAs circulate in the bloodstream and that such circulating miRNAs are remarkably stable. This has raised the possibility that miRNAs may be probed in the circulation and can serve as novel diagnostic markers. Although the precise cellular release mechanisms of miRNAs remain largely unknown, the first studies revealed that these circulating miRNAs may be delivered to recipient cells, where they can regulate translation of target genes. In this review, we will discuss the nature of the stability of miRNAs that circulate in the bloodstream and discuss the available evidence regarding the possible function of these circulating miRNAs in distant cell-to-cell communication. Furthermore, we summarize and discuss the usefulness of circulating miRNAs as biomarkers for a wide range of cardiovascular diseases such as myocardial infarction, heart failure, atherosclerosis, hypertension, and type 2 diabetes mellitus. (*Circ Res*. 2012;110:483-495.)**

Key Words: plasma microRNAs ■ biomarkers ■ review ■ paracrine communication ■ cell-derived microparticles ■ exosomes

Only a decade ago, microRNAs (miRNAs) were discovered in mammals as a large class of evolutionarily conserved small noncoding RNAs. miRNAs regulate gene expression at the posttranscriptional level by targeting the 3′ untranslated region of mRNA transcripts.[1] By influencing protein translation, miRNAs have emerged as powerful regulators of a wide range of biological processes. It has been quickly recognized that miRNAs can be efficiently inhibited for prolonged periods by antisense technologies, which has fueled a growing interest in the inhibition of specific miRNAs as a feasible therapeutic option for selected cardiovascular diseases.[2]

In 2008, it was discovered that miRNAs are also present in blood, where they were detected in plasma, platelets, eryth-

Original received August 15, 2011; revision received September 30, 2011; accepted October 14, 2011. In October 2011, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 15 days.

From the Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands.

Correspondence to Esther E. Creemers, PhD, or Yigal M. Pinto, MD, PhD, Heart Failure Research Center, Room L2-208-3, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, Netherlands. E-mail e.e.creemers@amc.uva.nl or y.pinto@amc.uva.nl

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.111.247452**

Non-standard Abbreviations and Acronyms	
AGO2	argonaute2
AMI	acute myocardial infarction
AUC	area under the curve
CAD	coronary artery disease
cTnI	cardiac troponin I
HDL	high-density lipoprotein
MV	microvesicle
NPM1	nucleophosmin 1
ROC	receiver operating characteristic

rocytes, and nucleated blood cells.[3–5] Plasma miRNAs were found to be remarkably stable even under conditions as harsh as boiling, low or high pH, long-time storage at room temperature, and multiple freeze-thaw cycles.[3,5] In contrast to the stability of miRNAs endogenous to the sample, when synthetic miRNAs were added exogenously, they were quickly degraded by the high level of RNase activity in plasma.[3,5] This suggests that endogenous plasma miRNAs are protected in some manner to prevent their degradation. Recent reports have provided novel explanations for this surprising stability and have shown that miRNAs can be packaged in microparticles (exosomes, microvesicles, and apoptotic bodies)[6,7] or associated with RNA-binding proteins (Argonaute2 [Ago2])[8] or lipoprotein complexes (high-density lipoprotein [HDL])[9] to prevent their degradation. The presence of miRNAs in microparticles has also led to the intriguing idea that circulating miRNAs could have a function in cell-to-cell communication. This would suggest that miRNAs are selectively targeted for secretion in 1 cell and taken up by a distant, target cell, possibly to regulate gene expression. This is an intense area of investigation, and the first studies have recently revealed that miRNAs may indeed function as mediators of cell-to-cell communication.[6,10]

One of the major challenges in cardiovascular research is the identification of reliable biomarkers that can be measured routinely in easily accessible samples, such as plasma. Because of their stability in the circulation, miRNAs are currently being explored for their potential as biomarkers for cardiovascular disease. Thus far, distinctive patterns of circulating miRNAs have been found for myocardial infarction,[11] heart failure (HF),[12] atherosclerotic disease,[13] type 2 diabetes mellitus (DM),[14] and hypertension.[15] In the present review, we will discuss the available evidence of the cellular release mechanisms and the nature of the stability of miRNAs in the bloodstream (eg, microparticles, RNA-binding proteins, and HDL). Next, we will discuss the available evidence for a possible function of miRNAs in cell-to-cell communication. Finally, we will review the current knowledge about circulating miRNAs as putative biomarkers in cardiovascular disease.

Cellular Release and Stability of Extracellular miRNAs

As early as 1972, it was reported that intact extracellular RNA could be detected in plasma despite the presence of ribonucleases that were expected to destroy any freely circulating RNA.[16] It has therefore been suggested that this extracellular RNA, including miRNAs, is somehow shielded to prevent its degradation. As discussed in the sections below, evidence is now accumulating that miRNAs are protected against degradation by being packaged in lipid vesicles or by being associated with protein or lipoprotein complexes (Figure).

Plasma miRNAs in Microparticles

El-Hefnawy et al[17] were among the first to show that plasma RNA is protected from degradation by its inclusion in protein or lipid vesicles. Depending on their size and mode of release from cells, these particles are known as exosomes, microvesicles (MVs), or apoptotic bodies.[18] Exosomes are small vesicles (50–100 nm) that originate from the endosome and are released from cells when multivesicular bodies fuse with the plasma membrane. MVs are membranous vesicles that are larger (0.1–1 μm) than exosomes and are released from the cell through blebbing of the plasma membrane. Apoptotic bodies are the largest microparticles (0.5–2 μm) and are shed from cells during apoptosis.[18]

Several groups investigated the exosome content in greater detail in cultured cells. Valadi et al[7] revealed that mast cell–derived exosomes carry ≈121 different miRNAs. Some of these miRNAs were found at relatively higher levels in exosomes than in their donor cells, which implies an active mechanism by which selected miRNAs are promoted toward exosomes. Pegati et al[19] confirmed that cells can indeed select some miRNAs for cellular release while others are retained. Specifically, although 66% of the miRNAs were released in quantities that reflected their intracellular level, 13% of the miRNA species were selectively retained by the cell (and thus released at lower levels), whereas on the other hand, 21% of the miRNAs seemed to be actively released and appeared at disproportionately higher levels.[19] One of the most clearly actively released miRNAs was miR-451, of which >90% of the total mature miRNA population was exported into the extracellular space. Interestingly, precursors of mature miRNAs (pre-miRNAs) were also selectively released by those cells.[19] Furthermore, mesenchymal stem cells were shown to release miRNA-containing exosomes. Here, miRNAs were secreted predominantly as pre-miRNAs and not in their mature form.[20] The mechanism of miRNA secretion was further investigated in COS7 and HEK293 cells by Kosaka et al,[18] who showed that miRNAs are released through a ceramide-dependent secretory machinery. Ceramide, a bioactive sphingolipid whose biosynthesis is tightly controlled by neutral sphingomyelinase 2, triggers the secretion of exosomes. Blockade of neutral sphingomyelinase 2 by either a chemical inhibitor or by small interfering RNAs reduced secretion of miRNAs, whereas overexpression of neutral sphingomyelinase 2 increased the amount of extracellular miRNAs.[18] Further studies are needed to dissect the underlying mechanism that determines why some miRNAs are loaded into exosomes or multivesicular bodies for secretion whereas others are retained within the cell.

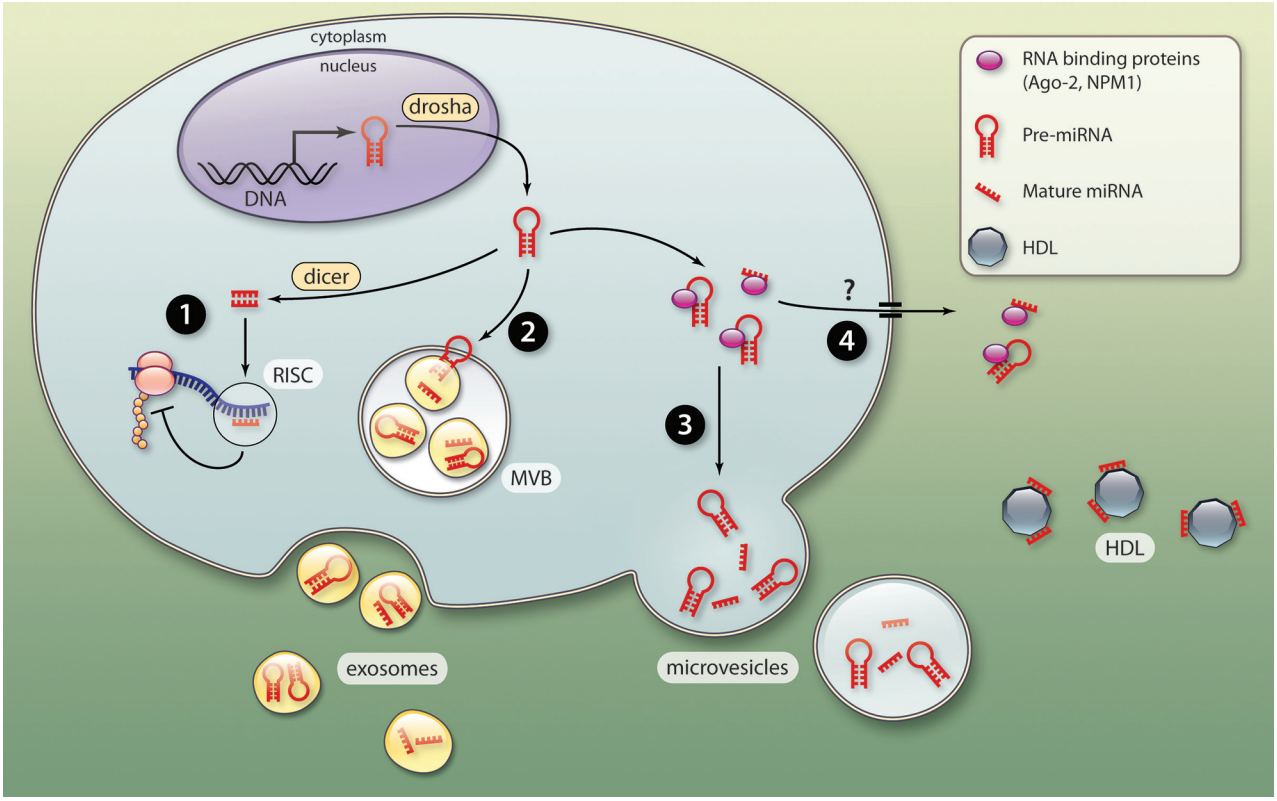

Figure. Cellular release mechanisms and extracellular transportation systems of miRNAs. In the nucleus, miRNAs are transcribed from DNA. A precursor hairpin miRNA (pre-miRNA) is formed after cleavage by the RNase III enzyme Drosha. After being transported into the cytoplasm, the pre-miRNA can be further cleaved into 19- to 23-nucleotide mature miRNA duplexes. One strand of the miRNA duplex can be loaded into the RNA-induced silencing complex (RISC), where it can guide the RISC to specific mRNA targets to prevent translation of the mRNA into protein (**1**). The other strand may be degraded or released from the cell through export mechanisms described below. In the cytoplasm, pre-miRNAs can also be incorporated into small vesicles called exosomes, which originate from the endosome and are released from cells when multivesicular bodies (MVB) fuse with the plasma membrane (**2**). Cytoplasmic miRNAs (pre-miRNA or mature miRNA) can also be released by microvesicles, which are released from the cell through blebbing of the plasma membrane (**3**). miRNAs are also found in circulation in microparticle-free form. These miRNAs can be associated with high-density lipoproteins or bound to RNA-binding proteins such as Ago2. It is not known how these miRNA-protein complexes are released from the cell. These miRNAs may be released passively, as by-products of dead cells, or actively, in an miRNA-specific manner, through interaction with specific membrane channels or proteins (**4**). Although pre-miRNAs have been detected in exosomes and microvesicles,[20] and mature miRNAs have been found in complex with Ago2[8] and HDL,[9] the exact proportion of mature and pre-miRNAs in the different extracellular compartments is not known. Illustration credit: Cosmocyte/Ben Smith.

miRNAs Associated With Protein Complexes

The proportion of miRNAs in the different cell-derived compartments is not yet settled. Currently, evidence is accumulating that the majority of miRNAs are not found inside vesicle but rather are bound to RNA-binding proteins.

Arroyo et al[8] used differential centrifugation and size-exclusion chromatography to systematically characterize circulating miRNA complexes in human plasma and serum. Their results support the hypothesis that at least 2 populations of circulating miRNAs exist: vesicle-associated and non–vesicle-associated miRNAs. They quantified 88 plasma miRNAs by quantitative real-time polymerase chain reaction (PCR) miRNA profiling. This revealed that vesicle-associated plasma miRNAs represent the minority, whereas potentially up to 90% of miRNAs in the circulation are present in a non–membrane-bound form. These non–vesicle-associated miRNAs were specifically destabilized by proteinase K digestion of plasma, which indicates the existence of an miRNA-protein complex as a mechanism for their stability in the RNase-rich circulation. Further characterization revealed that a significant portion of circulating miRNAs were associated with Ago2, which is known as the key intracellular effector protein of miRNA-mediated RNA silencing.[8] Interestingly, some miRNAs (eg, let-7a) were exclusively associated with vesicles, whereas others (eg, miR-122) were exclusively present in nonvesicle Ago2 complexes. This may reflect cell type–specific miRNA release mechanisms. For instance, the liver-specific miR-122 was detected only in protein-associated fractions, which suggests that hepatocytes release this miRNA through a protein carrier pathway. In contrast, miRNAs that are mainly associated with vesicles might originate from cell types that are known to generate vesicles, such as reticulocytes or platelets.[8]

In conditioned media of several cell lines, Wang et al[21] compared profiles of miRNAs in cell-derived vesicles (ie, exosomes and MVs) with vesicle-free miRNAs (ie, supernatant fraction after ultracentrifugation). In agreement with other studies, exported miRNAs were found in cell-derived vesicles; however, most of the miRNAs were independent of these vesicles. Because the miRNA profiles within and

outside these vesicles were strikingly different, it was hypothesized that they represented different export systems. Exposure of cultured HepG2 cells to various chemical inhibitors revealed that this miRNA export process is largely energy dependent but independent of the Golgi apparatus–mediated intracellular transport or exosome formation.[21] To examine the biochemical properties of these exported, vesicle-free miRNA complexes, Wang et al[21] performed mass spectrometry on medium of primary human fibroblasts. Twelve RNA-binding proteins were identified in the medium, among which were nucleophosmin 1 (NPM1), which has been implicated in the nuclear export of the ribosome, and nucleolin, a known NPM1-interacting protein.[22,23] Subsequent experiments showed that NPM1 can fully protect synthetic miRNA degradation by RNase A. It has been suggested that NPM1 may be involved in shuttling RNAs from the nucleus to the cytosol, and independent studies have also shown that NPM1 can be released into the extracellular space.[24] Together, this suggests that this mechanism may be relevant for miRNA export and stability; however, to the best of our knowledge, it is currently unknown whether NPM1 is also present in the cell-free circulation and binds to miRNAs in vivo.

Turchinovich et al[25] also reported that most extracellular miRNAs in blood plasma and cell culture are not associated with exosomes but are bound to Ago2 and, to a lesser extent, other Ago proteins. Using nanomembrane concentrators, they first determined that extracellular miRNA in plasma is associated with a protein complex of molecular weight between 50 and 300 kDa. Immunoprecipitations with 98-kDa Ago2 subsequently revealed that miRNAs were detected in Ago2 complexes. They also investigated NPM1 as a possible carrier for extracellular miRNAs and found NPM1 to have a molecular weight in plasma of ≈100 kDa, which is large for a 33-kDa protein. This probably reflects complex formation with other proteins such as nucleolin. Future studies including immunoprecipitations with anti-NPM1 in plasma should be performed to address whether NPM1 can bind circulating miRNAs.

In conclusion, the picture is emerging that a large portion of circulating miRNAs is associated with Ago2; however, it is unclear how miRNAs are exported from the cell. It has been proposed that the Ago2-miRNA complexes are passively released by death or apoptotic cells and remain in the extracellular space because of the high stability of the Ago2 protein. It is also possible that cell membrane–associated channels or receptors exist that allow for the specific release of these Ago2-miRNA complexes (Figure).

Circulating miRNAs Associated With HDL

Recently, it was shown that HDL can also transport endogenous miRNAs.[9] HDL particles have an average size of 8 to 12 nm, which makes them substantially smaller than exosomes. Furthermore, they contain lipids, such as phosphatidylcholine, that are known to form stable ternary complexes with nucleic acids.[26] They also contain as a main constituent apolipoprotein A-I, which has been used for the systemic delivery of small interfering RNAs in animal models.[27] Vickers et al[9] profiled miRNAs in purified HDL, low-density lipoprotein, and exosome pools from human plasma and revealed the following: (1) The HDL-miRNA profile was distinct from the exosome-miRNA profile; (2) the low-density lipoprotein–miRNA profile was more closely reflected by the exosome-miRNA signature than the HDL-miRNA profile; (3) HDL from familial hypercholesterolemia subjects had a higher concentration of miRNAs and contained more individual miRNAs than HDL from healthy subjects; (4) in 2 mouse models of atherosclerosis (ie, low-density lipoprotein receptor–null and apolipoprotein E–null mice on a high-fat diet), the HDL-miRNA profiles were distinct from those of control mice and showed a significant overlap with human familial hypercholesterolemia; and (5) mass spectrometry and Western blotting failed to identify previously identified RNA-binding proteins in the HDL fractions, such as Ago2 and NPM1. Collectively, these experiments have revealed that HDL carries miRNA signatures that differ between health and disease.

How HDL is loaded with miRNAs is not known exactly; however, biophysical studies suggest that HDL simply binds to extracellular plasma miRNA through divalent cation bridging.[9,26] The observation that reconstituted HDL injected into mice retrieved distinct miRNA profiles from normal and atherogenic models provides further evidence that HDL binds miRNAs in the circulation.[9]

One of the most abundant miRNAs in both human and mouse HDL is miR-223. This is also the most regulated HDL-associated miRNA in atherosclerosis because it is increased >3000-fold in human familial hypercholesterolemia.[9] Despite this dramatic increase in HDL miR-223, this miRNA was not identified as a putative circulating biomarker in plasma of patients with atherosclerosis.[13] This may relate to differences in disease characteristics (coronary artery disease [CAD] versus hypercholesterolemia) or sample properties (plasma versus HDL).

A Possible Role for miRNAs in Cell-to-Cell Communication

The stability of miRNAs in the circulation raises the intriguing possibility that they are taken up by distant cells to regulate their gene expression. Currently, the potential function of extracellular miRNAs is being studied intensively, and the first studies have confirmed that miRNAs may indeed function in cell-to-cell communication.

Communication Through Microparticles

One of the well-described functions of microparticles (MVs and exosomes) is to promote communication between the cells from which they are derived and their surrounding environment. Depending on the cell type from which these microparticles originate, they carry a range of bioactive molecules such as proteins, DNA, RNA, and miRNAs.[28] For instance, MVs derived from neutrophils are packed with cytokines.[29,30] Fusion of MVs with remote cells has been shown to result in the transfer of the MV contents and has been shown to affect tumor progression.[28,31] MVs secreted by endothelial cells have been implicated in angiogenesis,[32] and MVs from embryonic stem cells are capable of reprogramming hematopoietic progenitors.[33] The studies described

below have investigated the hypothesis that miRNAs in microparticles may be involved in cell-to-cell communication by mediating the repression of critical mRNA targets in distant recipient cells.

Along these lines, Zhang et al[10] identified miR-150 as an miRNA that is packaged into MVs by human blood cells and monocytic cells (THP1) in abundant quantities. Using DiI-labeled MVs and FITC-tagged miR-150 overexpression, they subsequently showed that these THP1-derived MVs directly delivered miRNAs to cultured endothelial cells, which reduced c-Myb protein levels, a validated target gene of miR-150.[10,34] Subsequently, an increase in endothelial cell migration rate was observed, which was blunted after anti–miR-150 treatment. Together, these results demonstrate that secreted miRNAs present in MVs can be delivered effectively to cultured cells, where they can function as endogenous miRNAs.[10] In vivo, intravenous injection of THP1-derived MVs in mice increased miR-150 in blood vessels at 6 hours after injection, whereas injection of MVs derived from 293T cells, which contain low levels of miR-150, failed to do so. Unfortunately, it was not shown whether these increased vascular miR-150 levels would reach levels high enough to significantly downregulate target genes. Finally, it was shown that plasma MVs from atherosclerotic patients had an increased level of miR-150. The authors concluded that secreted miR-150 may play a role in regulating endothelial cell function; however, because the described in vitro studies were mainly performed with miR-150–overexpressing cells and target-gene regulation was not shown in vivo, more research is warranted to validate this conclusion.

In the context of atherosclerosis, it has been reported that in addition to MVs, apoptotic bodies are released into the circulation, where they have been shown to inhibit atheroprogression. In a landmark study by Zernecke et al,[6] it was proposed that miR-126, carried by apoptotic bodies, is largely responsible for this protective effect via induction of the production of the chemokine CXCL12. In apolipoprotein E–null mice on an atherogenic diet, they showed that apoptotic bodies from wild-type mice protected against atherosclerosis, as evidenced by reduced infiltration of macrophages and an increased number of smooth muscle cells. In contrast, when they used apoptotic bodies from miR-126–deficient mice, CXCL12 expression in the carotid arteries failed to increase, and the protective effect on atherosclerosis was lost.[6] However, in this experimental atherosclerotic mouse model, the lumen of carotid arteries was experimentally incubated with relatively high concentrations of apoptotic bodies, and it is not known whether more "physiological" levels of apoptotic bodies would suffice to affect gene expression in a similar manner. Furthermore, additional controls are warranted to convincingly show that it is truly the miRNA component within the apoptotic bodies that mediates the observed atheroprotective effect.

Besides delivery of miRNAs by MVs to endothelial cells, in vitro studies have also shown that miRNAs can be transferred efficiently to mouse embryonic fibroblasts[35] and H9C2 cardiomyocytes.[20] In addition, Pegtel et al[36] have reported that miRNAs released in exosomes by Epstein-Barr virus–infected cells can be delivered to peripheral blood mononuclear cells, where they can suppress confirmed Epstein-Barr virus target genes.

Overall, the above studies have shown that microparticles are capable of functionally delivering miRNAs to recipient cells in vivo, but further research is needed to demonstrate to what extent this occurs under physiological and pathological conditions. The functional delivery of miRNAs to distant cells also may make microparticles ideal candidates as vehicles for such therapies. Future studies should address whether microparticles can be engineered or loaded with specific miRNAs to use as a novel therapeutic tool.

Communication Through HDL

To examine whether miRNAs transported on HDL alter gene expression in distant cells, Vickers et al[9] obtained HDL from patients with hypercholesterolemia and HDL from healthy subjects and used this to treat cultured hepatocytes. HDL from patients with hypercholesterolemia increased levels of miR-105 in these cells, whereas HDL from healthy subjects had no effect. HDL from familial hypercholesterolemia patients induced significant gene expression changes in hepatocytes, and microarray analysis and in silico target prediction revealed that 60% of the downregulated genes were putative targets of miR-105. This study provides evidence that HDL can deliver miRNAs to cells and alter gene expression. Nevertheless, some of the observed gene-expression changes from HDL treatment could also be related to differences in protein or lipid composition.[9]

In conclusion, the remarkable stability of miRNAs in the circulation is related to their packaging in microparticles and their association with Ago2 and HDL. In the future, it will be important to further clarify which of these compartments contain functional miRNAs. As demonstrated by Arroyo et al,[8] the majority of the circulating miRNAs appear to be associated with Ago2, and this raises the intriguing possibility that cells release a functional miRNA-induced silencing complex into the circulation, which suggests that these complexes may be poised to regulate gene expression in distant recipient cells. However, it remains unknown whether miRNA-Ago2 complexes can be taken up by recipient cells in the first place.

The miRNAs that were found to be associated with HDL were ≈22 nucleotides in length, which indicates these are mature miRNAs.[9] It appears unlikely that those mature miRNAs play any biological function, because only pre-miRNAs and not mature miRNAs can be loaded in the RNA-induced silencing complex. In addition, these HDL-bound miRNAs were not associated with Ago2, and it is therefore a mystery how these mature miRNAs could have regulated gene expression in recipient cells in culture, as shown by Vickers et al.[9] Chen et al[20] showed that mesenchymal stem cells secrete exosomes that contain primarily miRNAs in the precursor form instead of the mature form. These precursor miRNAs were not found to be associated with Ago2 or Dicer, but this does not preclude them from exerting biological effects in recipient cells, because these precursor miRNAs may potentially make use of the RNA-induced silencing complex of recipient cells.[20]

Circulating miRNAs as Biomarkers

The stability of miRNAs in plasma and the ease by which miRNAs can be detected in a quantitative manner by methods such as real-time PCR and microarrays have sparked great interest in the use of circulating miRNAs as clinical biomarkers. An ideal biomarker fulfills a number of criteria, such as accessibility through noninvasive methods; a high degree of specificity and sensitivity; the ability to differentiate pathologies, allowing early detection; sensitivity to relevant changes in the disease; a long half-life within the sample; and the capability for rapid and accurate detection.[37] Because circulating miRNAs are able to fulfill a number of those criteria, since 2009, more than 10 groups have reported on the use of miRNAs as circulating biomarkers for diagnosis or prognosis of cardiovascular diseases such as myocardial infarction, HF, atherosclerosis, hypertension, and DM. Although many of those studies still require replication in multiple independent study populations, the picture emerges that some plasma miRNAs are quite specific for cardiovascular pathologies and not only may be useful for diagnostic and monitoring purposes but also may provide much needed intermediate end points for clinical trials. An overview of these studies is discussed below and summarized in the Table.

Plasma miRNAs After Myocardial Infarction

Several groups have studied the hypothesis that heart-specific miRNAs leak into the circulation during an acute myocardial infarction (AMI) and can be used to detect and monitor myocardial injury. Four cardiac miRNAs (miR-208a, miR-499, miR-1, and miR-133) are found to be consistently elevated in plasma of AMI patients within hours after the onset of infarction.[11,38–45] Of these 4 miRNAs, miR-208a, which is encoded by an intron of the αMHC gene, is to the best of our knowledge the only heart-specific miRNA.[46] The other 3 miRNAs (miR-499, miR-1, and miR-133), besides being highly expressed in the heart, are also expressed in skeletal muscle.[47,48]

In a study by Wang et al,[11] 66 patients with chest pain were evaluated clinically by use of biochemical markers (cardiac troponin I [cTnI] threshold >0.1 ng/mL), complaints (acute ischemic-type chest pain), electrocardiographic changes, and coronary angiography. They were compared with 30 control subjects. Confirmed AMI patients (33 subjects) were separated from non-AMI patients with chest pain (33 subjects) by cTnI and coronary angiogram. Real-time PCR revealed that miR-1, miR-133a, and miR-499 were detected at significantly higher levels in plasma from AMI patients than from the non-AMI or healthy control group. Strikingly, miR-208a could not be detected in plasma samples from healthy control subjects or in the non-AMI patients with chest pain but was readily detectible in 91% of the AMI patients. The sensitivity and specificity of these miRNAs in the diagnosis of AMI were evaluated by side-to-side comparisons of receiver operating characteristic (ROC) curves. MiR-208a showed a superior ROC curve, with an area under the curve (AUC) of 0.965 (95% confidence interval 0.920–1.000), which was very similar to the AUC of cTnI (0.987). In the early stages of an AMI, miR-208a may even have advantages over cTnI, because it was shown that miR-208a could be detected in plasma of all patients within 4 hours of the onset of symptoms, whereas cTnI was only detected in 85% of patients at this early stage.[11] However, D'Alessandra and colleagues[40] were unable to detect elevated levels of circulating miR-208a in all AMI patients. As will be discussed below, this may relate to the relatively late time points at which plasma samples were collected (on average ≈9 hours after the onset of AMI symptoms, which is well after miRNA levels have peaked in the bloodstream). Alternatively, it may also relate to the relatively low levels of miR-208 in the blood compared with other muscle-enriched miRNAs, even after AMI, which may be below the detection limit of the protocol used.[40]

Corsten et al[38] investigated plasma levels of the other miR-208 family member, miR-208b, in AMI patients. MiR-208b is encoded by an intron of βMHC and is therefore coexpressed with βMHC in both skeletal muscle and heart. When plasma miR-208b levels in 32 AMI patients were compared with those in 36 patients presenting with atypical chest pain without cardiac cause, a 1600-fold increase in miR-208b levels was found in plasma of AMI patients, and ROC curves revealed an AUC of 0.944 (95% confidence interval 0.863–1.000).

To assess the exact time course of miRNA release after AMI, 3 groups have experimentally induced myocardial infarction in rodents by coronary artery ligation.[11,40,45] Wang et al[11] collected blood samples from rats at 6 time points (0, 1, 3, 6, 12, and 24 hours) after coronary artery ligation and showed that miR-208a was undetectable before surgery or in sham-operated control rats but increased significantly to detectable levels as early as 1 hour after ligation, peaked at 3 hours, and then began to decrease, returning to undetectable levels at 24 hours after AMI. Interestingly, cardiac hypertrophy was not sufficient for miR-208 to be released from cardiac myocytes, as was shown in a rat model of hypertensive cardiac hypertrophy in salt-sensitive Dahl rats on a high-salt diet.[39] Levels of miR-1, miR-133a, and miR-499 did increase significantly after AMI; however, their increase was slightly slower and peaked at 6 hours, and levels were still elevated at 24 hours after AMI.[11,45] D'Alessandra et al[40] monitored miRNAs in a mouse model of myocardial infarction and found miR-499-5p to be an extremely sensitive indicator of cardiac damage; it closely paralleled the increase in troponin I after coronary artery ligation. However, troponin I peaked at the 6-hour time point, whereas miR-499-5p exhibited a slower time course, reaching its peak only at 24 hours after myocardial infarction.[40] The same group also investigated the time course of miRNA release in a small number of patients (n=8) and compared these levels with cTnI. Similar to the rodent AMI models, plasma miR-1 and miR-133 peaked slightly earlier than troponin I, within 3 hours after AMI, whereas miR-499-5p exhibited a slower time course and peaked after troponin I, at ≈12 hours after myocardial infarction.[40]

An unexpected but important observation by 2 independent groups is that tissue injury during the surgical procedure of coronary artery ligation also effectively releases muscle-borne miRNAs into the blood.[11,42] Both groups convincingly

Table. Circulating miRNAs as Diagnostic Markers in Cardiovascular Disease

Disease	Study Design	miRNA Biomarkers	Source	Age/Sex Differences Between Groups?	Multivariate Analyzed?	Reference
AMI	33 AMI; 33 non-AMI with chest pain; 30 healthy subjects	miR-1, miR-133a, miR-208a, miR-499	Plasma	No	No	11
AMI	33 AMI; 17 healthy subjects	miR-1, miR-133a, miR-133b, miR-499-5p	Plasma	Controls >10 y younger	Age corrected	40
AMI	32 AMI; 36 non-AMI with AP	miR-208b, miR-499	Plasma	No	No	38
AMI	14 AMI; 10 healthy subjects	miR-499	Plasma	Controls >25 y younger	No	43
AMI	93 AMI; 66 healthy subjects	miR-1	Plasma	No	No	44
AMI	29 AMI; 42 nonacute CAD	miR-1, miR-133a	Serum	Sex differences	No	42
AMI	31 AMI; 20 healthy subjects	miR-1	Serum	Age- and sex-matched controls used	No	45
HF	30 HF; 20 non-HF with dyspnea; 39 healthy subjects	miR-423-5p, miR18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p	Plasma	Healthy subjects >10 y younger†	Age and sex corrected	12
HF	33 HF; 20 healthy subjects	miR-499, miR-122	Plasma	Controls >40 y younger	No	38
HF	10 HF; 17 asymptomatic controls	miR-126 Not changed: miR-122, miR-499	Plasma	Controls >25 y younger	No	53
HF	15 HF; 10 healthy subjects	miR-499 is not changed	Plasma	Controls >20 y younger	No	43
CAD	67 CAD; 31 healthy subjects	Increased: miR-133, miR-208a Decreased: miR-126, miR-17, miR-92a, miR-155, miR-145	Plasma	Controls >30 y younger and sex differences	No‡	13
CAD	12 CAD; 12 healthy subjects	miR-140, miR-182	Whole blood	No	No	59
CAD	50 CAD; 20 healthy subjects	Increased: miR-135 Decreased: miR-147	PBMC	Age- and sex-matched controls used	No	70
HYP	194 Hypertensive patients; 97 healthy subjects	HCMV-miR-UL112, miR-296-5p, let-7e	Plasma	No	Age, sex, BMI, DM, lipids, CHD	15
DM	80 DM; 80 controls	miR-126, miR-15a, miR-29b, miR-223, miR-28-3p	Plasma	Age- and sex-matched controls used	Yes§	14
DM	162 Impaired glucose tolerance; 580 controls	miR-126‖	Plasma	Unknown	Yes§	14

AMI indicates acute myocardial infarction; AP, angina pectoris; CAD, coronary artery disease; HF, heart failure; PBMC, peripheral blood mononuclear cells; HYP, hypertension; BMI, body mass index; DM, type 2 diabetes mellitus; and CHD, coronary heart disease.

In miR-18b, the asterisk refers to the mature miRNA that derives from the minor arm (less frequently found in the cell) of the pre-miR-18 hairpin.

†There were no age differences when HF patients were compared with non-HF patients with dyspnea.

‡Significant correlations were found between plasma miR-155 and age (negative correlation) and sex.

§Multivariate adjustments for BMI, waist/hip ratio, smoking, social status, alcohol, physical activity, and C-reactive protein.

‖miR-126 is the only miRNA that was validated in a prospective population of 822 individuals (Bruneck study).[14] In that study, loss of miR-126 was observed before the onset of overt DM and was associated with vascular complications.

demonstrated that sham-operated rats and mice both showed a similar increase of plasma miR-1 and miR-133a as seen in the AMI group at the 3- and 6-hour time points. MiR-208a, which is expressed in a cardiac-specific fashion, was not elevated in plasma of sham-operated rats, and miR-499, which is expressed at high levels in the heart and at low levels in skeletal muscle, increased only slightly in plasma of sham-operated rats.[11]

To specifically investigate whether skeletal muscle damage can lead to an increase of miRNA plasma levels, D'Alessandra et al[40] used a mouse model of acute hind-limb ischemia and measured plasma miRNAs at 6 and 24 hours after femoral artery dissection. Interestingly, miR-1 and miR-133a plasma levels failed to increase in this model. In contrast, Muzino et al[49] showed that serum levels of miR-1 and miR-133a do increase in the dystrophin-deficient muscular dystrophy mouse model, *mdx*, as well as in canine X-linked muscular dystrophy in the Japanese dog model, which indicates that muscle pathologies may also be associated with release of these miRNAs.

In conclusion, accumulating evidence indicates the usefulness of muscle miRNAs as stable blood-based biomarkers for AMI. In the early phase after AMI (<3 hours), miR-1, miR-133a, and more particularly miR-208a may even be more sensitive than the classic biomarker cTnI, because these miRNAs achieve their peak before cTnI. This earlier miRNA peak suggests a faster leakage of miRNAs than cTnI from damaged cardiomyocytes because of differential release kinetics. In this regard, cTnI is mainly bound to myofibrils,[50] whereas miRNAs are probably bound to protein complexes in the cytosol, the latter allowing a faster release from damaged cells.[11] Interestingly, not all myocardial miRNAs leak into the circulation with the same kinetics. The slower release of miR-499 into the bloodstream compared with several other myocardial miRNAs may suggest that the different miRNAs are bound to different proteins within cells and in the circulation. Strikingly, miR-30c and miR-24 are strongly expressed in the heart, even more strongly than miR-208, but their levels in the bloodstream still failed to increase after AMI.[40] The release kinetics, as well as the stability of the miRNAs in the circulation, probably reflect the biochemical properties of the protein to which the miRNA is bound.

Which miRNA Is the Best Candidate for AMI Diagnosis?

Because miR-208a is the only heart-specific miRNA and is therefore minimally affected by noncardiac tissue injury, this miRNA appears to be the superior miRNA for the diagnosis of AMI. Evidence suggests that miR-208a release peaks before cTnI in plasma, and ROC curves show that sensitivity and specificity are very similar between miR-208a and cTnI.[11] However, ROC curves are useful but not very informative in studies with small sample sizes, so larger studies are needed to more precisely evaluate the added clinical value of measurement of each circulating miRNA. Several studies have shown that plasma miR-499 also accurately diagnoses AMI patients,[11,38,40,43] with ROC curves revealing an AUC of 0.822 (95% confidence interval 0.717–0927)[11] and 0.918 (95% confidence interval 0.942–0.995),[38] which is lower than the AUC found for miR-208a (AUC 0.965, 95% confidence interval 0.920–1.000). Advantages of miR-499 may relate to the more robust detection than with miR-208a and to the slower release of miR-499 into the bloodstream than with other miRNAs and cTnI, which suggests that this miRNA may have additional value in diagnosis at later time points after AMI.

MiR-1 and miR-133 may also serve to diagnose AMI, although there are conflicting results on the correlation with muscle creatine kinase levels and cTnI levels in plasma.[11,40,44,45] Because these miRNAs are also highly expressed in skeletal muscle and possibly other tissues, their plasma levels may be affected by underlying pathologies of other organs. In this regard, miR-1 and miR-133a have also been found to increase in the bloodstream of patients with lung cancer[5,51] and colorectal cancer.[52]

Taken together, the combined assessment of the 2 myomiRs miR-208a and miR-499 may provide an attractive signature to diagnose both acute and very recent cardiac injury (miR-208), whereas miR-499 may still trace signs of myocardial injury that occurred longer ago.

Plasma miRNAs in HF

To explore whether circulating miRNAs can be used as biomarkers in patients with HF, our laboratory has performed miRNA arrays on plasma of 12 healthy control subjects and 12 HF patients.[12] Subjects with recent cardiac ischemia or infarction were excluded, so results were less likely to be influenced by cardiac cell death. Indeed, no increases in miR-1, miR-208a, miR-208b, or miR-499 were found in plasma of HF patients selected in this manner.[12] From these arrays, 16 candidate miRNAs were selected and validated in a second group of patients, consisting of 50 case subjects with complaints of dyspnea, 30 of whom were later diagnosed to have HF and 20 of whom were diagnosed to have dyspnea attributable to other, non-HF causes. Seven miRNAs were validated in the plasma of patients with HF (miR-423-5p, miR-18b*, miR-129-5p, miR-1254, miR-675, HS_202.1, and miR-622), among which mature miR-423-5p was most strongly related to the clinical diagnosis of HF, with an ROC showing an AUC of 0.91 (95% confidence interval 0.84–0.98). Comparison of HF case subjects not only to control subjects but also to dyspneic patients who were free of HF allowed us to distinguish miRNAs that were upregulated more generally in subjects with dyspnea. An example is miR-675, which appeared to be an attractive candidate when HF case subjects were compared with healthy control subjects but appeared to be generally upregulated in dyspnea and was not specific for HF. Interestingly, the abundance of some miRNAs was related to disease severity, because it was found that levels of circulating miR-423-5p and miR-18b* were higher in subjects with the poorest ejection fraction and New York Heart Association classification. Although miR-423-5p and miR-18b* may be attractive novel biomarkers specific for HF, several important questions remain unanswered. First, it is still not clear which cell type is responsible for the release of miR-423-5p into plasma. The observation that miR-423-5p is upregulated in human failing myocardium suggests that the increased plasma levels are derived from the myocardium.[12] However, this is still uncertain, because other miRNAs known to be locally expressed at high levels in the failing myocardium were not found in this study. Second, it is unanswered how this miRNA is released into the circulation. Is there a specific secretory pathway, or does the release result from cell death and subsequent passive release of cellular content into the extracellular space? It has been

shown that in the circulation of healthy subjects, miR-423-5p is specifically bound to Ago2 complexes independent of vesicles. It is currently unknown whether this is also the case in HF patients.[8] Third, given that miR-423-5p is bound to Ago2 in plasma, might it fulfill biological functions outside the cell?

Fukushima et al[53] analyzed the expression of 3 miRNAs in plasma of 10 HF patients and 17 asymptomatic control subjects. They found that the endothelium-specific miR-126 was negatively correlated with age (R^2=0.52, P=0.0006), brain natriuretic peptide (r^2=0.25, P=0.0003), and New York Heart Association class (P=0.0001), which indicates that this miRNA could be used as a biomarker for HF. Lower levels of miR-126 were also found in atherosclerotic CAD[13] and in patients with type 2 DM[14] and may reflect the condition of vascular endothelial cells in HF patients. More definitive studies in HF are needed to define the most promising miRNA biomarkers in this patient group.

miRNA Detection in Atherosclerotic CAD

Atherosclerosis, the main underlying cause of cardiovascular disease, is regarded as an inflammatory disease of the vessel wall, characterized by endothelial activation and accumulation of lipid in infiltrated macrophages that starts the process of plaque formation and narrows the vessel lumen, whereas acute rupture of an unstable plaque can cause complete obstruction.[54] The progression of CAD is highly variable, and it is of clinical importance to identify subjects with unstable plaques at risk of acute coronary syndromes. Plasma proteins such as C-reactive protein and fibrinogen, as well as leukocyte counts, have been investigated intensively as possible biomarkers for plaque vulnerability; however, meta-analyses have demonstrated that their diagnostic and prognostic value for future cardiovascular complications is limited.[55] In addition, the existing imaging techniques mainly detect the end stage of the disease. Therefore, innovative and reliable biomarkers for atherosclerosis and plaque stability are much needed. So far, 3 studies have addressed the potential of circulating miRNAs as biomarkers for CAD.

Fichtlscherer et al[13] were the first to investigate levels of plasma miRNAs in stable atherosclerotic disease in humans. Patients in their study had angiographic documentation of CAD and were excluded if they had an impaired ejection fraction, HF, unstable CAD, or AMI, so that plasma miRNAs were less likely to be influenced by major cardiac cell death and less likely to be related to plaque instability. Using miRNA arrays, circulating miRNA signatures were studied in plasma of a small group of subjects (8 CAD patients versus 8 healthy volunteers), and this resulted in the identification of 46 downregulated miRNAs and 20 significantly upregulated miRNAs in plasma of stable CAD patients. Most of the differentially expressed miRNAs were validated by quantitative real-time PCR in a second, independent cohort comprising 36 patients with documented CAD and 17 control subjects. Interestingly, most of the identified and validated downregulated miRNAs were abundantly expressed in the vessel wall, in particular in endothelial cells. These included miR-126, miR-92a, and miR-17. In addition, both the vascular smooth muscle cell–enriched miR-145 and the inflammatory cell–related miR-155 were also found to be significantly downregulated in these CAD patients. Although cardiac muscle–enriched miRNAs (miR-208 and miR-133) tended to be higher in the initial discovery cohort of patients with stable CAD, this increase did not attain statistical significance, and in the validation cohort, no increase in miR-208 or miR-133 was seen. It is conceivable that in their initial discovery cohort of stable CAD patients, some patients may have been included with subclinical myocardial injury. Patients with acute myocardial injury were excluded, but the report did not state whether this required a negative troponin test, so it cannot be excluded that some patients with CAD may have had subclinical, acute, or chronic myocardial damage. Nevertheless, the lack of significant increases in circulating miR-208 and miR-133 appears to be in line with the idea that these miRNAs only probe AMI, so that slight elevations may be caused by a small number of patients with a low degree of subclinical myocyte injury.[13]

A decrease in the level of circulating endothelial miRNAs in the plasma of patients with stable CAD is a rather surprising observation, because the development of atherosclerotic lesions is known to be associated with endothelial activation, and this in turn has been shown to induce the release of microparticles,[56] known to contain miRNAs. These reduced concentrations of endothelial miRNAs may reflect the observed decrease in the number of circulating endothelial cell progenitors reported to occur in patients with CAD.[57] Alternatively, Fukushima et al[53] showed that plasma concentrations of miR-126 were negatively correlated with age. Because subjects of the CAD cohort of Fichtlscherer et al[13] were reported to be ≈30 years older than the healthy control subjects, this may also provide a plausible explanation for the reduced levels of miR-126. The underlying mechanism for the loss of miR-126 in the circulation with age is currently unknown but may relate to deteriorating perfusion, decreased renewal of endothelial cells, or cellular aging, which may lead to a reduction in the release of miRNAs.[53]

The observation that the inflammation-associated miR-155 is downregulated in plasma of subjects with CAD was also not directly expected, because it is evident that atherosclerosis is associated with inflammation of the vessel wall, and inflammatory cells are the major source of miR-155.[13] An explanation may lie in the differences in cohort characteristics, because an inverse correlation was found between circulating miR-155 levels and age, and it was shown that miR-155 levels are lower in males than females.[13] Subjects in the CAD cohort were substantially older than the healthy control subjects and comprised more males than females, which may explain the loss of miR-155 in plasma. Follow-up studies in larger groups of patients and control subjects are needed to confirm these findings and to unravel the underlying mechanism of the loss of these miRNAs in the circulation of CAD patients and in the aging population. It would also be interesting to determine whether candidate miRNA levels correlate with some of the more established plasma biomarkers, such as C-reactive protein, interleukin-6, and lipoprotein-associated phospholipase A2.[58]

Taurino et al[59] investigated miRNA expression in whole blood of CAD patients. In 10 CAD patients, whole blood

gene-expression profiling was performed before and after subjects completed an exercise-based rehabilitation program after surgical coronary revascularization. Microarray analysis of miRNA expression in whole blood revealed 2 miRNAs, miR-140 and miR-182, that differed between CAD and control subjects. Interestingly, expression of miR-92 increased after cardiac rehabilitation, which is in line with the study by Fichtlscherer et al,[13] in which miR-92 was reduced in plasma of CAD patients.

Hoekstra et al[70] also studied the miRNA signature in the circulation of CAD patients; however, they used peripheral blood mononuclear cells. In a study group of 20 control subjects, 25 subjects with stable angina pectoris, and 25 subjects with unstable angina pectoris, real-time PCR analysis of 157 different miRNAs revealed that in peripheral blood mononuclear cells of both patient groups, circulating miR-135a was increased 5-fold, whereas miR-147 was decreased 4-fold compared with healthy control subjects. Interestingly, miR-147 previously has been reported to be associated with changes in the inflammatory capacity of immune cells by repressing tumor necrosis factor-α and interleukin-6.[61] This suggests that peripheral blood mononuclear cells of CAD have an altered miRNA repertoire, possibly shifting to a more proinflammatory phenotype. Patients with unstable angina pectoris could be distinguished from stable patients on the basis of relatively high levels of 3 circulating miRNAs (miR-134, miR-198, and miR-370), which suggests that the miRNA signatures can be used to identify patients with a vulnerable plaque.[60] However, this study is limited by the relatively small number of individuals in the 3 cohorts and the fact that RNA of 8 to 9 patients was pooled for the real-time PCR assays, which negatively impacts the power of the screen. In addition, candidate miRNAs were not validated in a second independent cohort.

In conclusion, initial studies fuel the notion that circulating miRNAs may become helpful tools in the diagnosis and prognosis of patients with CAD, but large-scale studies are needed to determine the true potential of circulating miRNAs as biomarkers.

Plasma miRNAs in Type 2 DM

Type 2 DM is characterized by chronic elevations of blood glucose levels and insulin resistance and is one of the major risk factors for cardiovascular disease.[60] Zampetaki and colleagues[14] performed miRNA profiling in 80 type 2 DM patients in the Bruneck study[61] and compared these with 80 age- and sex-matched control subjects. Extensive network analysis revealed a unique plasma miRNA signature for DM, which included reduced levels of miR-126, miR-15a, miR-29b, and miR-223 and elevated levels of miR-28-3p. Intriguingly, a reduction in the level of some of these miRNAs (miR-126, miR-15a, and miR-223) was already detectable years before the manifestation of diabetes. Among these miRNAs, the endothelial cell–derived miR-126 was most consistently associated with DM, which is interesting because miR-126 was also one of the identified downregulated miRNAs in atherosclerotic CAD.[13] MiR-126 has been shown to play an important role in maintaining endothelial cell homeostasis and vascular integrity by facilitating vascular endothelial growth factor signaling.[62,63] These findings suggest that this unique plasma miRNA signature may become a valuable tool to predict microvascular and macrovascular complications.

Plasma miRNAs in Essential Hypertension

A specific miRNA signature has also been identified in plasma of hypertensive patients.[15] Curiously, one of the successfully validated miRNAs appeared to be a human cytomegalovirus (HCMV)–encoded miRNA, hcmv-miR-UL112, which suggests a novel link between HCMV infection and essential hypertension. By measuring HCMV titers, Li et al[15] subsequently showed that HCMV virus titers were also substantially higher in the hypertension group than in control subjects (1870 versus 54 copies per milliliter of plasma, respectively). A significant correlation was found between HCMV virus titers and hcmv-miR-UL112 levels in hypertensive patients ($P=0.003$). A possible causal link was recently suggested in an animal study in which the infection of mice with mouse cytomegalovirus resulted in elevated blood pressure.[64] Altogether, miRNA profiling in plasma of hypertensive patients reveals the possible involvement of HCMV in the pathogenesis of essential hypertension and suggests potential therapeutic targets. However, because of the high degree of variation in the abundance of hcmv-miR-UL112 between patients, it remains to be seen whether this miRNA can be used as a biomarker for the diagnosis of hypertension.

Future Perspectives

miRNAs as Biomarkers

Current circulating biomarkers for cardiovascular disease are based on specific proteins, such as troponins and natriuretic peptides. The development of new protein-based biomarkers is often rather cumbersome because of the complexity of protein composition in blood, the diversity of posttranslation modifications, the low abundance of many proteins, and the difficulties in developing assays for high-sensitivity detection.[37] Detection of blood-based biomarkers is usually based on antibodies, which may exhibit cross-reactivity with other proteins.

In that respect, circulating miRNAs offer many features to make them an attractive class of biomarkers. They are stable; their sequences are evolutionarily conserved; miRNA expression is often tissue or pathology specific; and because they are detected by real-time PCR, assays can be highly sensitive and specific. However, there are also challenges associated with the detection of circulating miRNAs that still need to be addressed. One of the challenges relates to the low amount of total RNA in blood, which makes it virtually impossible to measure the concentration and quality of the isolated RNA. As a consequence, it is of crucial importance to precisely normalize detected miRNA values for variances based on the amount of starting material and miRNA extraction. This has been tried by seeking a "housekeeping " circulating RNA. Some reports use U6 or other miRNAs (eg, miR-16) as a housekeeping RNA; however, the levels of these RNAs often change under pathological conditions. Mitchell et al[3] reported a spiked-in normalization approach in which 3 synthetic

Caenorhabditis elegans miRNAs (without homology to human miRNAs) were added during the purification procedure and used for data normalization. This worked well in their hands; however, these synthetic miRNAs may be less stable than endogenous miRNAs when added to plasma. Cheng et al[45] report that plasma volume is the best factor with which to standardize the amount of input miRNA. The amount of molecules per volume of plasma or serum is also used as the standard to evaluate blood levels of other molecules. Future studies are warranted to systematically characterize the different normalization methods to find the best way to reproducibly measure miRNAs in plasma. In this regard, it is very possible that plasma miRNAs from microparticles, HDL, or unbound miRNA require a different normalization procedure.

Next-Generation Sequencing

The development of next-generation sequencing technologies has recently offered an unprecedented scale and depth of miRNA profiling. These technologies have the potential to be used to discover novel miRNA species and are not biased by thermodynamics, a drawback of quantitative PCR and microarray platforms.[65] The first studies to systematically characterize miRNAs in serum using next-generation sequencing were reported recently. Interestingly, this revealed that the major fraction of all small RNA sequences in serum consisted of miRNAs (<30 nt).[5] Two studies, both using Solexa sequencing, identified ≈100 different miRNAs in serum, and both studies revealed unique expression profiles in patients with different types of cancer.[5,51]

Next-generation sequencing also revealed that mature miRNAs have an extensive degree of variation at the terminal nucleotides, primarily at the 3′ side of the miRNA. This population of so-called isomiRs may derive from imprecise and alternative cleavage of Dicer and Drosha during pre-miRNA processing and from posttranscriptional additions of uridines and adenosines.[66,67] Although the biological relevance of these miRNA isoforms is not known precisely, these variations might influence miRNA half-life, subcellular localization, and miRNA target specificity, especially for 5′ end variations.[66,68] In addition, this heterogeneity also affects the accuracy of miRNA detection by traditional techniques such as microarrays and real-time PCR. Furthermore, next-generation sequencing revealed that in several instances, the most abundant miRNA species do not match the sequence listed in public databases such as miRBase, which suggests that probe or primer design may not be optimal for all miRNAs.[65]

IsomiRs have not yet been catalogued in plasma or serum, but these data sets are awaited with great interest. Interestingly, dynamic changes in isomiR populations have been described in Drosophila development, which may also suggest that certain isomiR populations may also be physiology and pathology specific.[66] Perhaps they eventually will provide more specific biomarkers than the common miRBase variants.

Clinical Application

The potential of circulating miRNAs as blood-based biomarkers for cardiovascular disease is promising, and initial candidate miRNAs as biomarkers for myocardial infarction, HF CAD, hypertension, and type II DM have been proposed. Importantly, it is to be expected that combining multiple miRNAs into an miRNA profile may provide greater accuracy than can be expected from the assessment of a single miRNA. Advances in technology platforms for miRNA detection, such as microarrays and next-generation sequencing, have allowed for the simultaneous interrogation of the complete small RNA repertoire. Indeed, microarray studies followed by extensive network analysis have recently provided evidence that a panel of miRNAs or an miRNA signature has a better potential to offer sensitive and specific diagnostic tests.[14,69] With only ≈100 different miRNAs present in plasma, profiling the complete 100 circulating miRNAs may provide a comprehensive analysis of pathologies in multiple organs.

In conclusion, the identification of stable circulating miRNAs challenges a number of concepts and launches a new generation of potential biomarkers. It challenges the concept of confined local, intracellular actions of miRNAs and suggests that they may have roles distant from the cell from which they originate, much like peptidic hormones in an endocrine system. However, fundamental questions about their transportation, distant actions, and feedback on these actions must be answered before one can further shape the idea that circulating miRNAs are parts of an miRNA-based mobile messaging system.

This launches a new generation of potential biomarkers, biomarkers for which the assay can be developed with relative ease, at relatively low expense, but with potentially unrivaled specificity and sensitivity. These assays could be designed to probe a large number of circulating miRNAs, if not all of them, more readily than protein assays. This could drastically change the use and interpretation of circulating biomarkers as we now know them.

Sources of Funding

This work was supported by the Dutch Heart Foundation (NHS2007-B077) and the Center for Translational Molecular Medicine, projects TRIUMPH (01C-103) and INCOAG (01C-201).

Disclosures

Dr Pinto is a cofounder of and holds less than 5% equity in ACS Biomarker BV, a company that commercializes cardiovascular biomarkers.

References

1. Ambros V. The functions of animal microRNAs. *Nature*. 2004;431:350–355.
2. van Rooij E, Marshall WS, Olson EN. Toward microRNA-based therapeutics for heart disease: the sense in antisense. *Circ Res*. 2008;103:919–928.
3. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105:10513–10518.
4. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, Hatton CS, Harris AL. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008;141:672–675.

5. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Zen K, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997–1006.
6. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal*. 2009;2:ra81.
7. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9:654–659.
8. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011;108:5003–5008.
9. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13:423–433.
10. Zhang Y, Liu D, Chen X, Li J, Li L, Bian Z, Sun F, Lu J, Yin Y, Cai X, Sun Q, Wang K, Ba Y, Wang Q, Wang D, Yang J, Liu P, Xu T, Yan Q, Zhang J, Zen K, Zhang CY. Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol Cell*. 2010;39:133–144.
11. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J*. 2010;31:659–666.
12. Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, Pinto YM. MiR423–5p as a circulating biomarker for heart failure. *Circ Res*. 2010;106:1035–1039.
13. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Roxe T, Muller-Ardogan M, Bonauer A, Zeiher AM, Dimmeler S. Circulating microRNAs in patients with coronary artery disease. *Circ Res*. 2010;107:677–684.
14. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res*. 2010;107:810–817.
15. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, Ma X, Bond Lau W, Rong R, Yu X, Wang B, Li Y, Xiao C, Zhang M, Wang S, Yu L, Chen AF, Yang X, Cai J. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. *Circulation*. 2011;124:175–184.
16. Kamm RC, Smith AG. Nucleic acid concentrations in normal human plasma. *Clin Chem*. 1972;18:519–522.
17. El-Hefnawy T, Raja S, Kelly L, Bigbee WL, Kirkwood JM, Luketich JD, Godfrey TE. Characterization of amplifiable, circulating RNA in plasma and its potential as a tool for cancer diagnostics. *Clin Chem*. 2004;50:564–573.
18. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem*. 2010;285:17442–17452.
19. Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM. Selective release of microRNA species from normal and malignant mammary epithelial cells. *PLoS One*. 2010;5:e13515.
20. Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res*. 2010;38:215–224.
21. Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res*. 2010;38:7248–7259.
22. Borer RA, Lehner CF, Eppenberger HM, Nigg EA. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell*. 1989;56:379–390.
23. Maggi LB Jr, Kuchenruether M, Dadey DY, Schwope RM, Grisendi S, Townsend RR, Pandolfi PP, Weber JD. Nucleophosmin serves as a rate-limiting nuclear export chaperone for the mammalian ribosome. *Mol Cell Biol*. 2008;28:7050–7065.
24. Nawa Y, Kawahara K, Tancharoen S, Meng X, Sameshima H, Ito T, Masuda Y, Imaizumi H, Hashiguchi T, Maruyama I. Nucleophosmin may act as an alarmin: implications for severe sepsis. *J Leukoc Biol*. 2009;86:645–653.
25. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res*. 2011;39:7223–7233.
26. Janas T, Yarus M. Specific RNA binding to ordered phospholipid bilayers. *Nucleic Acids Res*. 2006;34:2128–2136.
27. Kim SI, Shin D, Choi TH, Lee JC, Cheon GJ, Kim KY, Park M, Kim M. Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol Ther*. 2007;15:1145–1152.
28. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. *J Cell Sci*. 2010;123(part 10):1603–1611.
29. Koppler B, Cohen C, Schlondorff D, Mack M. Differential mechanisms of microparticle transfer to B cells and monocytes: anti-inflammatory properties of microparticles. *Eur J Immunol*. 2006;36:648–660.
30. Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, Plachy J, Stangassinger M, Erfle V, Schlondorff D. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med*. 2000;6:769–775.
31. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10:1470–1476.
32. Morel O, Toti F, Hugel B, Freyssinet JM. Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. *Curr Opin Hematol*. 2004;11:156–164.
33. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*. 2006;20:847–856.
34. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, Rajewsky N, Bender TP, Rajewsky K. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007;131:146–159.
35. Yuan A, Farber EL, Rapoport AL, Tejada D, Deniskin R, Akhmedov NB, Farber DB. Transfer of microRNAs by embryonic stem cell microvesicles. *PLoS One*. 2009;4:e4722.
36. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Wurdinger T, Middeldorp JM. Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A*. 2010;107:6328–6333.
37. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. *Mutat Res*. 2011;717:85–90.
38. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B. Circulating microRNA-208b and microRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet*. 2010;3:499–506.
39. Ji X, Takahashi R, Hiura Y, Hirokawa G, Fukushima Y, Iwai N. Plasma miR-208 as a biomarker of myocardial injury. *Clin Chem*. 2009;55:1944–1949.
40. D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, Rubino M, Carena MC, Spazzafumo L, De Simone M, Micheli B, Biglioli P, Achilli F, Martelli F, Maggiolini S, Marenzi G, Pompilio G, Capogrossi MC. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J*. 2010;31:2765–2773.
41. Bostjancic E, Zidar N, Stajer D, Glavac D. MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. *Cardiology*. 2010;115:163–169.
42. Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, Imai M, Tamura T, Kita T, Kimura T. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate the existence of myocardial damage. *Circ Cardiovasc Genet*. 2011;4:446–454.
43. Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, Nonogi H, Iwai N. Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clin Chem*. 2010;56:1183–1185.
44. Ai J, Zhang R, Li Y, Pu J, Lu Y, Jiao J, Li K, Yu B, Li Z, Wang R, Wang L, Li Q, Wang N, Shan H, Li Z, Yang B. Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction. *Biochem Biophys Res Commun*. 2010;391:73–77.
45. Cheng Y, Tan N, Yang J, Liu X, Cao X, He P, Dong X, Qin S, Zhang C. A translational study of circulating cell-free microRNA-1 in acute myocardial infarction. *Clin Sci (Lond)*. 2010;119:87–95.

46. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science*. 2007;316:575–579.
47. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ Jr, Olson EN. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. *Dev Cell*. 2009;17:662–673.
48. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*. 2006;38:228–233.
49. Mizuno H, Nakamura A, Aoki Y, Ito N, Kishi S, Yamamoto K, Sekiguchi M, Takeda S, Hashido K. Identification of muscle-specific microRNAs in serum of muscular dystrophy animal models: promising novel blood-based markers for muscular dystrophy. *PLoS One*. 2011;6:e18388.
50. Wu AH, Feng YJ. Biochemical differences between cTnT and cTnI and their significance for diagnosis of acute coronary syndromes. *Eur Heart J*. 1998;19(suppl N):N25–N29.
51. Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C, Shen H. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol*. 2010;28:1721–1726.
52. Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut*. 2009;58:1375–1381.
53. Fukushima Y, Nakanishi M, Nonogi H, Goto Y, Iwai N. Assessment of plasma miRNAs in congestive heart failure. *Circ J*. 2011;75:336–340.
54. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317–325.
55. Lobbes MB, Kooi ME, Lutgens E, Ruiters AW, Lima Passos V, Braat SH, Rousch M, Ten Cate H, van Engelshoven JM, Daemen MJ, Heeneman S. Leukocyte counts, myeloperoxidase, and pregnancy-associated plasma protein a as biomarkers for cardiovascular disease: towards a multi-biomarker approach. *Int J Vasc Med*. 2010;2010:726207.
56. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension*. 2006;48:180–186.
57. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res*. 2004;95:343–353.
58. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. 2003;111:1805–1812.
59. Taurino C, Miller WH, McBride MW, McClure JD, Khanin R, Moreno MU, Dymott JA, Delles C, Dominiczak AF. Gene expression profiling in whole blood of patients with coronary artery disease. *Clin Sci (Lond)*. 2010;119:335–343.
60. Nathan DM. Long-term complications of diabetes mellitus. *N Engl J Med*. 1993;328:1676–1685.
61. Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Meigs JB, Bonadonna RC, Muggeo M. Population-based incidence rates and risk factors for type 2 diabetes in white individuals: the Bruneck study. *Diabetes*. 2004;53:1782–1789.
62. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell*. 2008;15:261–271.
63. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell*. 2008;15:272–284.
64. Cheng J, Ke Q, Jin Z, Wang H, Kocher O, Morgan JP, Zhang J, Crumpacker CS. Cytomegalovirus infection causes an increase of arterial blood pressure. *PLoS Pathog*. 2009;5:e1000427.
65. Lee LW, Zhang S, Etheridge A, Ma L, Martin D, Galas D, Wang K. Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA*. 2010;16:2170–2180.
66. Fernandez-Valverde SL, Taft RJ, Mattick JS. Dynamic isomiR regulation in Drosophila development. *RNA*. 2010;16:1881–1888.
67. Ebhardt HA, Tsang HH, Dai DC, Liu Y, Bostan B, Fahlman RP. Meta-analysis of small RNA-sequencing errors reveals ubiquitous post-transcriptional RNA modifications. *Nucleic Acids Res*. 2009;37:2461–2470.
68. Borel C, Antonarakis SE. Functional genetic variation of human miRNAs and phenotypic consequences. *Mamm Genome*. 2008;19:503–509.
69. Liu R, Zhang C, Hu Z, Li G, Wang C, Yang C, Huang D, Chen X, Zhang H, Zhuang R, Deng T, Liu H, Yin J, Wang S, Zen K, Ba Y, Zhang CY. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. *Eur J Cancer*. 2011;47:784–791.
70. Hoekstra M, van der Lans CA, Halvorsen B, Gullestad L, Kuiper J, Aukrust P, van Berkel TJ, Biessen EA. The peripheral blood mononuclear cell microRNA signature of coronary artery disease. *Biochem Biophys Res Commun*. 2010;394:792–797.

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2012;110:483-495
doi: 10.1161/CIRCRESAHA.111.247452
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571