

microRNA-based diagnostics and therapy in cardiovascular disease – Summing up the facts

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Abstract: Circulating microRNAs (miRNAs) are discussed as potential disease-specific biomarkers in cardiovascular disease. Their diagnostic value has been examined in numerous studies and animal models with respect to coronary artery disease (CAD) and myocardial infarction (MI) and the prognostic abilities of circulating miRNAs in risk stratification of future disease have been evaluated. Various miRNAs are described to complement protein-based biomarkers or classical risk factors in the diagnosis of CAD or MI and even represent potential new biomarkers in the discrimination of unstable angina pectoris (UAP). Signatures consisting of sets of multiple miRNAs seem to improve the predictive power compared to single miRNAs. Furthermore, the emerging field of miRNA-based therapeutics has reached cardiovascular research. The first promising *in vitro* results are raising hope for future clinical application. However, methods and material used for RNA isolation, miRNA detection and normalization steps still lack ways of standardization and need to be considered carefully. This article reviews the current knowledge of miRNAs in cardiovascular disease focusing on CAD and MI and will provide an overview regarding the use of circulating miRNAs as biomarkers and potential therapeutic targets in the field of CAD.

Keywords: Artery disease; biomarker; cardiovascular disease; circulating; coronary; diagnostic; microRNA (miRNA); myocardial infarction (MI); prognostic; risk stratification

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In 1993 the research community began to discover the function and importance of small RNAs and their power in controlling gene expression in humans (1) and termed them “microRNAs” (miRNAs) (2). Since then miRNAs have attracted scientists’ attention for their promising role as diagnostic and prognostic biomarkers in various diseases (3-5) and have been described as potential gene specific therapeutic targets in disease modeling (6).

In cardiovascular disease, miRNAs are considered disease-specific biomarkers with defined groups of circulating miRNAs being quantitatively altered in certain disease entities such as myocardial infarction (MI), atherosclerosis, coronary artery disease (CAD), heart failure, atrial fibrillation, hypertrophy and fibrosis (7-10).

In this review article we are discussing the value of

cardiovascular miRNAs with a focus on CAD and MI and provide an overview about their use as acute and prognostic biomarkers as well as therapeutic agents for cardiovascular diseases.

miRNA—a wrap-up of what they are

miRNAs are a class of abundant, non-coding RNAs of 19-25 nucleotides length recognized as evolutionarily conserved regulatory RNA molecules (11,12). miRNAs negatively regulate gene expression at the post-transcriptional level by translational repression or degradation of messenger RNAs (mRNAs), thus affecting a variety of cell processes (13-15). At the time of writing 2,588 mature homo sapiens miRNAs are listed in “miRBase” (16). Computational prediction

of target mRNAs suggests that more than 60% of all mammalian protein-coding genes are conserved targets of miRNAs (17), while every single miRNA has target sites in hundreds of different genes (18). miRNAs reveal tissue-specific expression (19) but also show concentration-dependent effects in pathologically affected organs and tissues (20).

The discovery of microRNAs as promising new biomarkers in the field of cardiovascular disease and CAD in particular has ignited great expectations. miRNA quantification showed organ- and cell-specific expression patterns of certain miRNAs (19) and *in vitro* findings suggest groups of miRNAs being specifically up and down regulated in different fields of cardiovascular disease (21), while polymorphisms in the miRNA regulatory pathway—so called miRSNPs—have shown association with different types of disease (22-24). Therefore, miRNAs fulfill several criteria of an ideal biomarker. Stability in the circulation, tissue- and pathology-specific regulation as well as high sensitivity and specificity suggest that miRNAs' applicability as biomarkers for cardiovascular disease might even exceed protein-based biomarkers (25,26). The field of miRNA research has created potential new means of biomarker-based risk stratification for cardiovascular events and promising data have been collected in a large-scale prospective clinical study (27).

Biomarkers in the field of CAD

The diagnosis of stable CAD with an elevated risk for future MI is based on invasive coronary angiography. Studies with CAD patients have revealed altered levels of certain miRNAs compared with healthy controls (7,21,28) highlighting their potential use as diagnostic biomarkers in CAD.

The gain in sensitivity of newly developed high sensitive cardiac troponin (hs-cTn) assay (29,30) comes along with an increase in false positive measurements caused by non-coronary cardiac pathologies (31). Moreover the early diagnosis of acute myocardial infarction (AMI) is still limited by the delayed release of troponin. A biomarker combining high sensitivity and specificity is needed to earlier detect AMI and to further reduce AMI mortality (32). Furthermore, there is still a lack of possibilities to differentiate troponin-negative unstable angina pectoris (UAP) from non-coronary chest pain (NCCP). In this acute setting of cardiovascular disease numerous studies have shown that miRNAs can serve as biomarkers for MI (33-35).

miRNAs in CAD and MI

By modulating different pathways implicated in the regulation of endothelial cell (EC) functions microRNAs and their key regulatory enzyme Dicer play a major role in the function of ECs (36). Dicer-knock down induced dysregulation of miRNAs that are highly expressed in ECs (let-7, mir-21, mir-126, mir-221, and mir-222) results in impaired EC proliferation (37) going along with decreased levels of inflammatory chemokines and cytokines such as IL-8, IL-1 β as well as chemokine ligands 1 and 3 (38). These observations emphasize the involvement of miRNAs in the development of atherosclerosis and CAD.

During the process of plaque development different sets of miRNAs have been found in different stages of plaque progression (39) and miRNA dysregulation plays a crucial role in the destabilization and rupture of atherosclerotic plaques (40).

Vascular miRNAs

In vitro studies revealed several miRNAs to specifically influence angiogenesis and the integrity of the endothelial monolayer (37). In particular, miR-27b and miR-130a were detected to stimulate and miR-221 and miR-222 to inhibit angiogenesis (41,42) linking them to the pathogenesis of atherosclerosis and CAD. In fact, miR-221 and -222 have been described to regulate other miRNAs in an *in vitro* study, amongst them miR-223 (43). The development of atherosclerotic plaque involves inflammation, which is strongly influenced by cholesterol homeostasis (44). In a mouse model Vickers *et al.* were able to show that cholesterol biosynthesis as well as uptake and efflux are regulated by miR-223 (45) suggesting miR-223 to be a potential mediator of atherosclerosis. Moreover in the same study the authors describe elevated levels of miR-223 inside thrombocytes of patients with diagnosed atherosclerosis. At the same time angiogenesis associated miRNA-106b, -25, -92a and -21 were described as significantly increased in microparticles from atherosclerotic plaques (46). Especially miR-92a had been proven to be a key regulator of angiogenesis in a mouse model as well as in human umbilical vein derived endothelial cells (47). Interestingly though, different studies found pro-atherosclerotic functions of miR-92a and an overexpression of miR-92a in ECs blocked angiogenesis *in vitro* and *in vivo* (47,48).

miR-143 and -145 are the most highly expressed microRNAs in vascular smooth muscle cells (VSMCs),

controlling differentiation and function (49). In an *in vitro* model their expression was found decreased during vascular stress suggesting their function in a modulated phenotype of VSMCs as observed in vascular disease (50). To emphasize their importance in atherosclerotic disease, miR-143 and -145 were identified as essential for neointima formation in response to vascular injury (51).

Beside miR-142 and -145, miR-126 was described as blood flow-sensitive miRNA, which regulates endothelial gene expression and is involved in endothelial dysfunction as well as atherosclerosis triggered by alterations of blood flow conditions (52) as they occur in CAD.

Myocardial miRNAs

miR-1 is the most abundant miRNA specific for cardiac and skeletal muscle and functions as a regulator of differentiation and proliferation during cardiogenesis (53) as well as a regulator of cardiomyocyte growth in the adult heart (54). At the same time miR-1 is considered pro-apoptotic in myocardial ischemia (55). In a rat model miR-1 levels significantly increased in the myocardium 12 hours after coronary artery occlusion (56). In human hearts of patients who had died of MI miR-1 was upregulated in remote myocardium as compared to infarcted tissue or healthy adult hearts (57).

miR-133, which is transcribed from the same chromosomal loci as miR-1, enhances myoblast proliferation and thus is involved in cardiomyocyte proliferations (58). Interestingly, though, while miR-1 supports apoptosis in oxidative stress, miR-133 was found to repress caspase-9 expression and thus has anti-apoptotic effects (55).

miR-21 is upregulated in cardiomyocytes shortly after initiation of ischemia before during cell death its concentration decreases (59). However, increased expression of miR-21 in fibroblasts enhances their proliferation (59) suggesting a role of miR-21 in remodeling.

As shown in a mouse model miR-208 is exclusively expressed in cardiomyocytes and consequently released during cardiomyocyte death in MI (60).

Members of the miR-29 family are downregulated in the region adjacent to MI areas in mice and humans (61). The authors discuss the role of miR-29 as a regulator in cardiac fibrosis and a potential target of tissue fibrosis.

Levels of miR-126 were up regulated in the non-infarcted areas after induced MI in rat hearts (62). At the same time a different group reported significantly reduced survival rates in miR-126 knock-out mice after permanent coronary

artery occlusion compared with wild-type mice (63). The authors hold defective angiogenesis in miR-126-null mice responsible for this effect. miR-126 is supposed to be mainly involved in the reparative phase after myocardial injury by enhancing angiogenesis (59).

The effect of ischemia-reperfusion on miRNAs in the rat heart was evaluated by Tang *et al.* (64). The authors found that levels of miR-1, miR-126 and miR-208 were increased while miR-21, miR-133 and miR-195 levels had decreased. Botjancic and colleagues analyzed cardiac tissue samples of 50 patients who had died from MI as well as eight formerly healthy trauma victims (65). The authors found miR-208 upregulated in infarcted tissue whereas miR-1, miR-133a/b were downregulated compared with healthy controls.

miRNAs and their use as circulating biomarkers in body fluids

Intracellular miRNAs have been proven to be regulators and mediators of the metabolism under pathological conditions (66). Intercellular miRNAs provide gene regulation and phenotypic control in physiological conditions (67). Having been stably detected in numerous body fluids including serum and plasma as well as saliva, tears and urine (25,26), the importance of miRNAs as extracellular messengers in cell to cell communication and their potential use as circulating biomarkers for cardiovascular disease has been recognized over the past few years (3,68).

Circulating miRNAs have also been detected in exosomes, microvesicles, and apoptotic bodies—so-called microparticles (69,70)—as well as in association with RNA-binding proteins (71) or in HDL lipoprotein complexes (72). These structural conditions are being held responsible for the extraordinary stability of miRNAs in circulating blood (25,73), which presents a prerequisite for the detection of miRNAs in body fluids. The release of miRNAs into extracellular compartments and thus into body fluids and especially into the blood stream has presented the possibility to non-invasively detect those circulating miRNAs and to use them as disease biomarkers. Numerous studies in the field of biomarker research are based on quantification of circulating miRNAs.

Circulating miRNAs in the diagnosis of acute MI

Subsequently to the identification of miR-208 to be exclusively expressed in the hearts of rats Ji *et al.* used an

isopropanol-induced MI model to show that miR-208 plasma levels significantly increased after MI (74). The authors found a comparable time course with cardiac troponin with respect to the detection limit and concluded that miR-208 might serve as a useful circulating biomarker in MI detection. Verification of these findings came from a different study group that found miR-208 plasma levels elevated in rats after coronary artery occlusion (75). In plasma levels of 407 patients with suspected ACS miR-208b and miR-499-5p were tested to successfully discriminate MI (76). Compared with troponin T, the discriminating power measured by AUC was considerable lower (cTnT =0.95; miR-208b =0.82; miR-499-5p =0.79).

On the basis of these findings Adachi *et al.* performed a miRNA array analysis in various human tissues to identify heart-specific miRNAs and found miR-499 almost specifically expressed in the heart (77), concluding that miR-499 might serve as an additional promising biomarker of MI. Similar results were reported in 92 elderly non ST segment elevation myocardial infarction (NSTEMI) patients. The authors found miR-499-5p levels more than 80-fold increased compared with 99 control persons as well as 81 patients with acute heart failure without MI (78). The power of miR-499-5p in discriminating NSTEMI from control was comparable to cTnT. More importantly, accuracy to differentiate NSTEMI from acute heart failure was higher for miR-499-5p than for cardiac troponins. This observation suggests that miRNAs might potentially be useful biomarkers helping to reduce cardiac troponins'—and especially hs-cTn—rate of false positive results in non-coronary heart disease.

In serum miR-1 was found to be increased by 200-fold with a peak six hours after induced MI in a rat model while levels correlated with infarction size and serum levels of creatine kinase MB (CK-MB) (79) while in a study with 93 acute MI patients and 66 healthy humans significantly higher miR-1 levels were detected in plasma samples of MI patients compared to controls (80). These findings were confirmed by Wang *et al.* in 33 acute MI patients compared with 30 healthy people as well as 16 patients with stable CAD and 17 patients with other cardiovascular disease (75). In a similar setting plasma miRNA levels of 444 ACS patients were measured and levels of miR-1, miR-133a and miR-208b were elevated in MI compared with patients with UAP (81). Interestingly levels of miR-1, miR-133a/b and miR-208b were independently associated with high sensitive troponin T levels. Li and colleagues also found miR-1, miR-133a, miR-208b and miR-499 plasma

levels elevated in 67 patients with acute MI compared to 32 healthy controls (82). At discharge from hospital the expression of the miRNAs had fallen to their baseline plasma levels. Moreover, ROC curve analyses revealed that those four miRNAs were non-inferior to troponin T with respect to prediction of MI.

These data indicate that several circulating miRNA show deregulated levels during ischemic conditions and MI and seem to be suitable biomarkers for the diagnosis of MI. *Table 1* gives an overview of circulating miRNAs dysregulated in MI.

Recently, in a meta-analysis Cheng *et al.* concluded that miRNAs are suitable for use as diagnostic biomarkers of MI (99). Fifteen studies with reported sensitivity, specificity and AUC analysis for MI were found while four studies did not report these findings. They found an overall sensitivity of 0.78, specificity of 0.82, and an AUC of 0.91 for overall miRNA analysis with respect to discrimination of MI. Subgroup analysis for the four most-reported miRNAs (miR-1, miR-133a, miR-208b and miR-499) confirmed these findings, whereby miR-1 showed particularly low sensitivity and specificity. Furthermore, the authors found a high correlation between miRNAs and other diagnostic biomarkers of MI such as cTnT, hs-cTnT as well as CK and CK-MB.

Compared with blood samples reports of miRNA quantification for diagnostic purposes of MI in other body fluids are rare. In 20 STEMI patients urine levels of miR-1 were found significantly elevated compared with 20 healthy controls, though (85). Worth noticing is the fact that urine miR-1 levels were reported strongly correlated to serum cardiac troponin I and CK-MB.

Circulating miRNAs in the diagnosis of stable CAD

One of the first groups to study human circulating miRNAs in CAD in a clinical setting was Hoeckstra *et al.* in 2010 (100). In 50 patients with invasively diagnosed CAD, 129 out of 157 screened miRNAs were expressed in peripheral blood mononuclear cells (PBMCs). miR-135a and -147 were found to be significantly elevated in CAD patients compared with healthy controls. Their findings pointed to the application of blood-based circulating miRNAs as biomarkers in the detection of CAD. Another early case control study has performed a gene expression profiling in whole blood of 163 patients with CAD compared with 63 healthy controls and found miR-140-3p, -182 levels reduced in CAD patients (101).

Table 1 Systematic overview of miRNAs dysregulated in MI								
miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference	
Single miRNAs								
miR-1	Plasma	MI =93; HC =66	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(80)	
	Plasma	MI =33; HC =17	qRT-PCR	Up-regulated in MI	Diagnostic	No	(83)	
	Plasma	MI =33; non-MI CAD =16; other CVD =17; healthy =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(75)	
	Plasma	MI =25; HC =11	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(84)	
	Serum	ACS =29; non-ACS =42	qRT-PCR	Up-regulated in ACS	Diagnostic	Yes	(34)	
	Plasma	ACS =444	qRT-PCR	Up-regulated in ACS	Diagnostic	No	(81)	
	Urine	MI =20; HC =20	qRT-PCR	Up-regulated in MI	Diagnostic	No	(85)	
	Plasma	MI =17; HC =25	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(86)	
	Plasma	MI =319; non-MI =88	qRT-PCR	Up-regulated in MI	Prognostic: mortality, heart failure	Yes	(76)	
	Plasma	MI =67; HC =32	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(82)	
	Plasma	MI =92; heart failure =81; healthy =99	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(78)	
	Plasma	MI =56; HC =28	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(87)	
	miR-21	Plasma	MI =92; heart failure =81; HC =99	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(78)
	miR-26a-5p	Serum	MI =31; HC =31	qRT-PCR	Down-regulated in MI	Diagnostic	Yes	(88)
miR-30a	Plasma	MI =18; HC =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(89)	
miR-92a	Plasma	MI =19; SAP =31; HC =7	qRT-PCR	Up-regulated in MI	Diagnostic	No	(90)	
miR-122	Plasma	MI =33; HC =17	qRT-PCR	Down-regulated in MI	Diagnostic	No	(83)	
miR-126	Plasma	MI =19; SAP =31; HC =7	qRT-PCR	Up-regulated in MI	Diagnostic	No	(90)	
	Plasma	MI =17; HC =25	qRT-PCR	Down-regulated in MI	Diagnostic	Yes	(86)	
miR-126-3p	Serum	MI =31; HC =31	qRT-PCR	Down-regulated in MI	Diagnostic	Yes	(88)	
miR-133a	Plasma	MI =33; HC =17	qRT-PCR	Up-regulated in MI	Diagnostic	No	(83)	
	Plasma	MI = 33; non-MI CAD =16; other CVD =17; HC =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(75)	
	Plasma	MI =19; SAP =31; healthy =7	qRT-PCR	Up-regulated in MI	Diagnostic	No	(90)	
	Plasma	MI =25; HC =11	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(84)	
	Serum	ACS =29; non-ACS =42	qRT-PCR	Up-regulated in ACS	Diagnostic	Yes	(34)	
	Plasma	MI =51; HC =28	qRT-PCR	Up-regulated in MI	Diagnostic	No	(33)	
	and whole blood	Plasma	ACS =444	qRT-PCR	Up-regulated in ACS	Diagnostic and prognostic	No	(81)
	Plasma	MI =67; HC =32	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(82)	
	Plasma	MI =92; heart failure =81; HC =99	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(78)	
	Plasma	MI =13; SAP =176; HC =127	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(91)	
	Plasma	MI =76; HC =110	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(92)	

Table 1 (continued)

Table 1 (continued)

miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference
miR-150	Plasma	MI =90	qRT-PCR Microarray	Down-regulated in pts with future increased remodeling	Prognostic: Remodeling	Yes	(93)
miR-150-3p	Serum	MI =31; HC =31	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(88)
miR-191-5p	Serum	MI =31; HC =31	qRT-PCR	Down-regulated in MI	Diagnostic	Yes	(88)
miR-195	Plasma	MI =18; HC =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(89)
miR-208a/b	Plasma	MI =32; NCCP =36	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(9)
	Plasma	MI =33; non-MI CAD =16; other CVD =17; HC =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(75)
	Plasma	MI =19; SAP =31; HC =7	qRT-PCR	Up-regulated in MI	Diagnostic	No	(90)
	Plasma	MI =25; HC =11	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(84)
	Plasma	ACS =444	qRT-PCR	Up-regulated in MI	Diagnostic and prognostic	No	(81)
	Plasma	MI =510; HC =87	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(94)
	Plasma	MI =319; non-MI =88	qRT-PCR	Up-regulation in future elevated risk for mortality, heart failure	Prognostic: mortality, heart failure	Yes	(76)
	Plasma	MI =67; HC =32	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(82)
miR-328	Plasma and whole blood	MI =51; HC =28	qRT-PCR	Up-regulated in MI	Diagnostic	No	(33)
mir-375	Plasma	MI =33; HC =17	qRT-PCR	Down-regulated in MI	Diagnostic	No	(83)
miR-423-5p	Plasma	MI =17; SAP =4; HC =5	qRT-PCR	Up-regulated in MI	Diagnostic	No	(95)
	Plasma	MI =92; heart failure =81; HC =99	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(78)
miR-486-3p	Serum	MI =31; HC =31	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(88)
miR-499	Plasma	MI =14; heart failure = 15; HC =10	qRT-PCR	Up-regulated in MI	Diagnostic	No	(77)
	Plasma	MI =33; HC =17	qRT-PCR	Up-regulated in MI	Diagnostic	No	(83)
	Plasma	MI =32; NCCP =36	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(9)
	Plasma	MI =33; non-MI CAD =16; other CVD =17; HC =30	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(75)
	Plasma	MI =19; SAP =31; HC =7	qRT-PCR	Up-regulated in MI	Diagnostic	No	(90)
	Plasma	MI =25; HC =11	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(84)
	Plasma	MI =510; HC =87	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(94)
	Plasma	MI =319; non-MI =88	qRT-PCR	Up-regulated in MI	Prognostic: mortality, heart failure	Yes	(76)
	Plasma	MI =67; HC =32	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(82)
	Plasma	MI =92; heart failure =81; HC =99	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(78)
	Unknown	MI =142, elderly NSTEMI	unknown	Up-regulated in future elevated risk for death	Prognostic: risk of death	No	(96)
let-7b	Plasma	MI =18; HC =30	qRT-PCR	Down-regulated in MI	Diagnostic	Yes	(89)
miR-663b	Plasma	MI =76; HC =110	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(92)
miR-1291	Plasma	MI =76; HC =110	qRT-PCR	Up-regulated in MI	Diagnostic	Yes	(92)

Table 1 (continued)

Table 1 (continued)

miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference
miRNA signature							
miR-126, miR-197, miR-223	Plasma	Clinical cohort	qRT-PCR	miR-126 up-regulated, miR-197 and miR-223 down-regulated in future MI	prognostic: MI	No	(27)
miR-1, miR-134, miR-186, miR-208, miR-223, miR-499	Serum	MI =117; SAP =182; HC =100	qRT-PCR	All up-regulated in MI	diagnostic	Yes	(97)
miR-16, miR-27a, miR-101, miR-150	Plasma	MI =150	qRT-PCR	miR-16 and miR-27a up-regulated, miR-101 and miR-150 down-regulated in MI	Prognostic: LV systolic function	No	(98)

qRT-PCR, quantitative real time polymerase chain reaction; MI, myocardial infarction; HC, healthy control; NCCP, non-coronary chest pain; SAP, stable angina pectoris; UAP, unstable angina pectoris; ACS, acute coronary syndrome; EPC, endothelial progenitor cell; PBSC, peripheral blood mononuclear cell; ROC, receiver operating curve.

Fichtlscherer *et al.* performed a miRNA screening and found the plasma and serum levels of a set of miRNAs altered in 36 patients with documented CAD compared with seventeen healthy controls (7). Interestingly, significantly reduced miRNAs were all either endothelial expressed (miR-126; miR-92a, miR-17) or smooth muscle (miR-145) and inflammatory cell-derived (miR-155). At the same time cardiac muscle enriched circulating miR-133 and miR-208 were elevated in CAD patients. MiR-126, miR-92a and miR-145 had previously been described as promising indicators for atherosclerosis in mouse and *in vitro* models (see above).

Weber *et al.* analyzed miRNA levels from whole blood samples of 10 CAD patients compared with 15 healthy controls (102). They found no quantitative changes of endothelial-derived miR-126 and miR-92a as reported before by Fichtlscherer *et al.* but did detect changes in miR-145 and miR-155—analogue to Fichtlscherer *et al.* The authors related the discrepancies to underlying differences of the blood samples. While Weber *et al.* used whole blood, Fichtlscherer *et al.* analyzed plasma and serum samples. These observations point towards large differences in miRNAs detected in different blood-derived material; these differences become important when considering miRNAs as potential biomarkers for diagnosis.

The finding of dysregulated miR-155 levels in CAD patients were confirmed by observations in 65 CAD patients

compared with 22 healthy controls that revealed miR-155 as well as miR-146a significantly induced in CAD patients (103). Elevated levels of miR-146a again were also reported in a recent Chinese case-control study involving 295 CAD patients and 283 healthy controls (104). Independently, significantly higher levels of the same pre-miR-146a variant were associated with CAD in South African patients (105). Finally, in a group of 40 CAD patients compared with healthy controls, platelet rich plasma was analyzed with regard to thrombocyte-derived miRNAs (106). Interestingly, none of the previously described and in atherosclerosis dysregulated platelet-associated miRNAs were described as quantitatively altered between the two groups. Instead miR-340* and miR-634* were upregulated in patients with CAD in two independent validation cohorts. These findings of dysregulated circulating human miRNAs indicate their promising utilization as biomarkers for diagnostic purposes in CAD. More detailed information is given in Table 2.

Circulating miRNAs in the diagnosis of stable and UAP

The definition of UAP includes normal blood values of cardiac troponin. Hence, the diagnosis of UAP is based on clinical evaluation while laboratory parameters are still lacking. Nevertheless, the diagnosis of UAP is crucial since

Table 2 Systematic overview of miRNAs dysregulated in CAD (including SAP and UAP)

miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference
Single miRNAs							
miR-1	Plasma	SAP =34; HC =20	qRT-PCR	Up-regulated in SAP	Diagnostic	Yes	(107)
miR-17	Plasma	CAD =36; HC =17	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(7)
miR-19a	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-21	Plasma	UAP =45; SA =31; HC =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-25	Plasma	UAP =45; SA =31; HC =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-29a	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-30e-5p	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-92a	Plasma	CAD =36; HC =17	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(7)
	EPC	CAD = n.a.; HC = n.a.	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(109)
	Plasma	UAP =45; SA =31; HC =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-106b	Plasma	UAP =45; SA =31; NA =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-126	Plasma	CAD = 36 HC = 17	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(7)
	EPC	CAD = n.a.; HC = n.a.	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(109)
	Plasma	SAP =34; HC =20	qRT-PCR	Up-regulated in SAP	Diagnostic	Yes	(107)
	Plasma, MV	SAP =181	qRT-PCR	Down-regulated in pts. with future cardiovascular events	Prognostic	No	(110)
miR-126*	Plasma	UAP =45; SA =31; HC =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-133	Plasma	CAD =36; HC =17	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(7)
miR-133a	Plasma	MI =13; SAP =176; HC =127	qRT-PCR	Up-regulated in CAD	Diagnostic	Yes	(91)
miR-135a	PBMC	UAP =25; SAP =25; HC =20	qRT-PCR	Up-regulated in SAP	Diagnostic	No	(100)
miR-140-3p	Whole blood	CAD =163; HC =63	Microarray	Up-regulated in CAD	Diagnostic	No	(101)
miR-145	Plasma	CAD =36; HC =17	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(7)
	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-146	PBMC	CAD =66; HC =33	qRT-PCR	Up-regulated in CAD	Diagnostic, Prognostic	No	(111)
miR-147	PBMC	SAP =25; HC =20	qRT-PCR	Down-regulated in SAP	Diagnostic	No	(100)

Table 2 (continued)

Table 2 (continued)

miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference
miR-150	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-155	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
	Plasma; PBMC	CAD =56; HC =54	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(112)
miR-181d	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-182	Whole blood	CAD =163; HC =63	Microarray	Up-regulated in CAD	Diagnostic	No	(101)
miR-199a	Plasma, MV	SAP =181	qRT-PCR	Down-regulated in future cardiovascular events	Prognostic	No	(110)
miR-208a	Plasma	CAD =36; HC =17	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(7)
miR-214	Plasma	CAD =40; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(113)
miR-221	PBMC	CAD =44; HC =22	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(114)
	EPC	CAD = n.a.; HC = n.a.	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(109)
miR-222	PBMC	CAD =44; HC =22	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(114)
	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
	EPC	CAD = n.a.; HC = n.a.	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(109)
miR-340*	Plasma	CAD =27; HC =40	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(106)
miR-342	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-378	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-484	Whole blood	CAD =10; HC =15	qRT-PCR	Down-regulated in CAD	Diagnostic	No	(102)
miR-485-3p	Plasma	SAP =34; HC =20	qRT-PCR	Up-regulated in SAP	Diagnostic	Yes	(107)
miR-590-5p	Plasma	UAP =45; SA=31; HC =37	qRT-PCR	Up-regulated in UAP	Diagnostic	Yes	(108)
miR-624*	Plasma	CAD =27; HC =40	qRT-PCR	Up-regulated in CAD	Diagnostic	No	(106)
miRNA signature							
SAP							
miR-1	Plasma	SAP =34; HC =20	qRT-PCR	All up-regulated in SAP	Diagnostic	Yes	(107)
miR-126							
miR-485-3p							

Table 2 (continued)

Table 2 (continued)

miRNA	Biomaterial	Groups	Detection method	Direction of alteration	Value as biomarker	ROC analysis	Reference
UAP							
miR-134, miR-198, miR-370	PBMC	UAP =25; SAP =25; HC =20	qRT-PCR	All up-regulated in UAP	Diagnostic	No	(100)
miR-1, miR126, miR-133a	Plasma	UAP =19; SAP =34; HC =20	qRT-PCR	All up-regulated in UAP	Diagnostic	Yes	(107)
miR-132, miR-150, miR-186	Serum	UAP =46; NCCP =63	qRT-PCR	Down-regulated in UAP	Differentiation UAP vs. NCCP	Yes	(115)

*, represents a different kind of miRNA. qRT-PCR, quantitative real time polymerase chain reaction; MI, myocardial infarction; HC, healthy control; NCCP, non-coronary chest pain; SAP, stable angina pectoris; UAP, unstable angina pectoris; ACS, acute coronary syndrome; EPC, endothelial progenitor cell; PBSC, peripheral blood mononuclear cell; ROC, receiver operating curve.

the risk for MI in these patients is elevated. Hoekstra *et al.* compared miRNA levels derived from PBMCs of 25 UAP patients with those of 25 patients diagnosed with stable angina pectoris (SAP) (100). They found a cluster of three miRNAs (miR-134, miR-198 and miR-370) that was overexpressed in UAP as compared with SAP suggesting that signatures of circulating miRNAs might be useful biomarkers to identify patients at risk for MI. These findings were in line with the description of microparticle derived pro-inflammatory miRNAs that were found to have discriminating power between stable CAD and UAP in the plasma of ten patients (116). However, D'Alessandra *et al.* failed to confirm these findings in plasma of 19 patients with UAP compared with 34 SAP patients (107). Addressing the same issue Zeller *et al.* performed a miRNA screening in patients with UAP compared with NCCP followed by a replication phase to verify the results. In a third step the authors evaluated miRNA levels in 46 UAP patients compared with 63 NCCP patients (115). A 3-miRNA-panel of miR-132, miR-150 and miR-186 showed a strong discriminating power between UAP and NCCP. Table 2 includes study results concerning miRNAs in SAP and UAP.

These are promising results on the way to close the diagnostic biomarker-based gap of differentiating stable CAD from ACS in troponin negative patients.

Value of miRNAs in prediction and prognosis of CAD and MI

Beside their diagnostic value circulating miRNAs are also

evaluated as potential predictive biomarkers with respect to cardiovascular disease and CAD and MI in particular.

Plasma levels of miR-133a and miR-208b were significantly associated with the risk of death in univariate and age- and gender-adjusted analyses of 444 ACS patients (81). Hoekstra *et al.* detected a miRNA signature consisting of three miRNAs (miR-134, -198 and -370) with the power to discriminate unstable from stable angina pectoris suggesting a potential to identify patients at risk for future cardiovascular events (100) pointing out the possibilities of miRNAs as a prognostic tool in clinical disease outcome. The finding of miR-370 as a potential prognostic biomarker in risk stratification for acute coronary events is backed up by analogous results in an earlier study in mice. Increased miR-370 expression levels had been found in response to induced ischemia (117). In a study involving 424 patients with suspected MI elevated plasma levels of miR-208b and miR-499-5p were strongly associated with increased risk of mortality or heart failure within 30 days (76). Although the association was lost after adjustment for troponin T the study provides evidence of an association of altered levels of circulating miRNAs with outcome after MI. Recently, in a screening and validation approach miR-652 was found to be significantly associated with post-MI readmission for heart failure while in combination with NT-proBNP and left ventricular ejection fraction this miRNA even improved risk stratification after MI (118). Similar results concerning the risk stratification of mortality or heart failure within 6 months after MI were described for miR-328 and miR-134 in a study population of 359 acute MI patients compared with 30

healthy volunteers (119).

So far, the only known prospective population-based cohort study examining the predictive value of circulating miRNAs with respect to MI included 820 people from the Bruneck study (27). In multivariable Cox regression analysis the authors found a signature of three miRNAs (miR-126, miR-197 and miR-223) involved in the prediction of MI. miR-126 levels were positively while miR-197 and miR-223 inversely associated with future MI. These are promising data for the use of circulating miRNAs in the field of population-based risk assessment for CAD.

MiRNA signatures in CAD

In addition to investigations of single miRNAs, several groups examined the potential of miRNA signatures in cardiovascular disease (Tables 1 and 2).

Ren *et al.* found a cluster of miRNAs consisting of the miR-106b/25 cluster, miR-17/92a cluster, miR-21/590-5p family, miR-451 as well as miR-126* to be dysregulated in a group of 13 patients with UAP and secured CAD compared with 13 NCCP patients (108). Importantly, even after adjustment for risk factors and the use of statins and anti-platelet drugs the levels of these circulating miRNAs were still independently associated with UAP.

The possibility that specific plasmatic miRNA signatures are capable of efficiently indicating the presence of SAP or UAP compared with healthy controls was supported by findings of D'Alessandra *et al.* In a cohort of 53 CAD patients with either SAP or UAP the authors found two sets of miRNAs with discriminating power against 20 healthy controls (107). Patients with SAP had higher levels of miR-1, miR-126 and miR-485-3p compared with the control group while miRNA levels in UAP patients were higher in case of miR-1, miR-126 and miR-133a when compared with healthy controls.

MiRNA signatures in acute MI

Li and colleagues examined serum samples from 117 acute MI patients, 182 AP patients and 100 healthy controls (97). After an initial screening of miRNA expression a validation phase revealed a signature consisting of six miRNAs (miR-1, miR-134, miR-186, miR-208, miR-223 and miR-499) with an AUC for the diagnosis of acute MI (AUC =0.83) that was higher than the AUC of cardiac troponin T (AUC =0.768) and CK-MB (0.709) when comparing MI and UAP. Compared with the use of each

of the single miRNAs the AUC value for the diagnosis of MI based on the miRNA signature was reported to be progressively higher suggesting superiority of miRNA signatures over single miRNAs. Similar results with respect to miRNA signatures come from Meder *et al.* In whole blood of patients with AMI they identified 121 miRNAs that were dysregulated compared with controls (120). The authors found a signature of 20 miRNAs that predicts AMI with better specificity and sensitivity as well as higher predictive power than each of the single miRNAs. In their study the use of the miRNA signature even allowed for an earlier diagnosis of AMI than cardiac troponin T. The same study group performed a whole-genome miRNA kinetic study in 18 AMI patients and performed serial measurements of miRNA expression levels (121). They were able to find a signature of seven miRNAs (miR-636, miR-7-1*, miR-380*, miR-1254, miR-455-3p, miR-566, and miR-1291) that was dysregulated early during AMI—five of them being part of the 20 miRNA signature discovered in the previous study. Table 1 provides an overview of miRNA signatures in MI.

Although these studies were performed with rather small sample sizes they emphasize the potential diagnostic power of miRNA signatures as biomarkers in AMI diagnostics.

miRNAs in disease treatment

The functional importance of miRNA-regulated networks is often revealed under stress conditions. At the same time miRNA loss of function may result in only slightly changed phenotypes (66). Difficulties in the use of therapeutically altering miRNAs lie in the fact that a single miRNA can affect expression of numerous proteins and genes while at the same time miRNA based regulation can involve a vast number of different miRNAs. miRNAs impose a relatively modest effect on their target, reflecting that individual mRNAs are targeted by multiple miRNAs while the cellular proteome might be able to compensate the absence of a single miRNA (122). Moreover, miRSNPs that can either modify the sequence of the miRNA itself or the miRNA-target site, are associated not only with cancer risk and outcome but also with altered treatment response (24). Nevertheless, promising results of *in vitro* testing and animal models indicate the possibility to successfully use miRNAs as therapeutic targets in disease.

Treatment options involving miRNAs imply the idea of specifically influencing the levels of miRNAs in certain diseases. This includes suppression of miRNAs as well as raising miRNA levels or substituting them by artificially

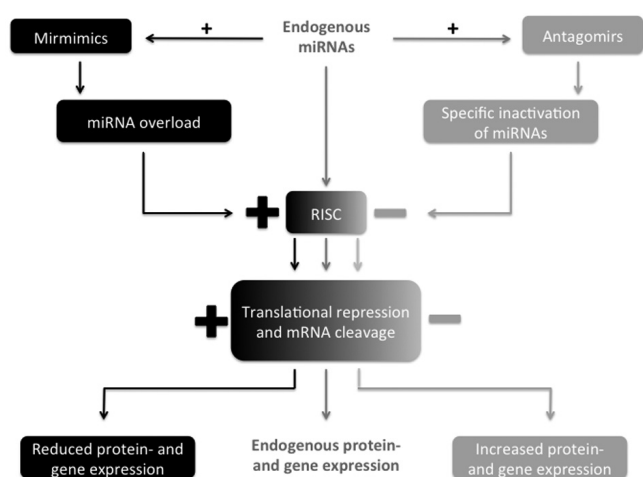


Figure 1 Use of miRNAs as therapeutics. Endogenous miRNAs (middle) activate the RNA-induced silencing complex (RISC) which in turn causes translational repression as well as mRNA cleavage and thus inhibits expression of genes and proteins. mirmimics (left) are artificial double-stranded miRNA-like RNA fragments that can be applied into the organism to additionally and specifically activate RISC and finally further reduce certain protein and gene expression. Antagomirs (right) are chemically engineered oligonucleotides specifically silencing endogenous miRNAs in order to reduce RISC activation and reduce translational repression/mRNA cleavage and in the end increase protein- and gene expression.

generated copies—depending on the mechanism of pathophysiology. The latter can be achieved by the miRNA mimic technology (miR-mimic) which is an approach for gene silencing by generating artificial double-stranded miRNA-like RNA fragments (123). These RNA fragments “mimic” endogenous miRNAs and bind specifically to its target mRNA—activating the RNA-induced silencing complex (RISC), down regulating specific mRNAs and thus induce gene suppression (*Figure 1*). As opposed to endogenous miRNAs miR-mimics function gene-specifically (123). While in cancer research miR-mimics have already largely been studied, however, in cardiovascular disease only few studies have been published so far.

Contrary, miRNA suppression can be obtained by antagomirs (124). Antagomirs are a class of chemically engineered oligonucleotides specifically silencing single endogenous miRNAs (125). They competitively inhibit specific miRNAs by binding to the target mature miRNA (124) and lead to a reduced activation of RISC and consequently to an upregulation of specific mRNAs and gene expression (*Figure 1*) (126).

Further approaches of reducing the amount and effect of miRNAs more or less specifically involve the use of miRNA sponges (also referred to as “target mimicry”), masking and erasers. Sponges are based on the mechanism of blocking the activity of a miRNA by means of a competitive inhibitor that contains a binding site for a miRNA family (127-129). Such inhibitors can be RNAs expressed transiently from transfected plasmids or stably from chromosomal insertions (127). By binding to the miRNA sponge with perfect or imperfect complementarity miRNAs’ effect on mRNA and gene expression is lessened because affected miRNAs cannot bind to their mRNA binding sites (124). As opposed to antagomirs, sponges are specific only to the seed region of a miRNA and thus are capable of blocking a whole family of miRNAs (130).

Masking describes an approach to hinder specific miRNAs from binding to their very binding site by masking the particular mRNA’s binding site of the target miRNA using perfectly matching oligonucleotides (131). As a consequence a smaller amount of miRNAs remains to bind to the target. Erasers are oligonucleotides complementary to a specific miRNA. By binding to the miRNA the eraser inhibits its endogenous function (124).

Studies including miR-mimics

Overexpression of miR-1 induces apoptosis and miR-1 levels strongly correlate with MI size in rats (55,64). By increasing miR-1 levels with the use of ischemic preconditioning a significant reduction of infarction size has been reported (79). miR-1 mimicry has shown that miR-1 regulates endothelin-1 in diabetes (132) but no reports are available specifically for MI.

After miR-21 levels were shown to be reduced in infarcted areas of rats after induced MI, Dong and colleagues were able to inhibit downregulation of this miRNA in infarcted areas by ischemic preconditioning and thus increasing miR-21 levels prior to infarction (62). At the same time transfection with adenovirus expressing miR-21 reduced cell apoptosis, decreased infarction size and improved left ventricular remodeling. Furthermore, intra cardiac injection of miR-21 along with miR-1 and miR-24 in mice was reported to reduce infarct size (133).

A different method of therapeutically raising miRNA levels was described by Huang and coworkers. In an experimental mouse model they showed that transplantation of mesenchymal stem cells overexpressing miR-1 into infarcted myocardium improved cardiac function by enhanced myocyte differentiation compared with control groups (134).

Studies including antagomirs

In a mouse model of MI a systemic application of an antagomir against miR-92a resulted in recovery of damaged tissue by enhanced blood vessel growth (47). Anti-miR-92a antagomirs could induce anti-atherosclerotic effects and block impairment of endothelial cells. An *in vitro* rat model of induced MI showed that silencing of miR-320 reduced cell death and apoptosis. The inhibition of miR-320 by use of antagomir-320 even led to a significant reduction of infarction size (135).

By using a miR-29 antisense inhibitor in a mouse ischemia-reperfusion model another study group was able to protect cardiomyocytes from injury (136). The authors even succeeded in reducing infarction size by application of antagomirs against miR-29a and miR-29c. miR-92a inhibition by systemic application of anti-miR-92a antagomirs led to enhanced functional recovery of damaged tissue, reduced apoptosis and improved MI size in a mouse model (47). Yang *et al.* showed that elimination of miR-1 by an antisense inhibitor in infarcted rat hearts had anti-arrhythmogenic effects after induced MI (56).

These studies provide evidence for the use miRNAs as a therapeutic target in CAD and MI and clinical trials are eagerly awaited.

Analytical considerations and standardization

Several confounding factors can influence the quality of miRNA data and need to be critically considered. Different sample preparation approaches and detection and normalization strategies are currently applied in a non-standardized fashion and application of universal reference material is required. Furthermore, variation caused by preanalytical and analytical factors can substantially influence these miRNA data (137). This can particularly be challenging in the clinical setting, as differences in sample collection, sample processing and assay performance in different clinical centers are to be expected. Therefore, to eliminate technical and analytical variability and to avoid artificial data generation, consensus on standard methods for all steps is demanded. Here, we briefly discuss the main influencing factors.

Methodological challenges and influencing factors

RNA isolation

Concentrations of miRNAs in body fluids are low, putting high expectations on the efficiency of their detection.

While co-extraction of minimal amounts of inhibiting factors can effect miRNA quantification, it is crucial to find and use methods of miRNA isolation with a maximum of reproducibility and reliability while several studies have described different qualities between the commercially used kits for miRNA isolation (138).

Detection methods

High throughput sequencing, real time PCR (qPCR) and microarrays are the three major quantification methods that are currently being used. While qPCR is considered the “gold standard” in the detection and quantification of defined sets of miRNAs for its high specificity and sensitivity, high-throughput sequencing of miRNAs is superior in the discovery of new miRNAs. Microarrays are considered the best option for standardized genome-wide assays on a larger scale (139). Over the past years universally applicable assays for miRNA quantification have been developed enabling scientists to easily perform qRT-PCR in quantifying miRNA levels. However, for example, in the diagnosis of AMI time is a crucial factor. So far miRNA quantification is still time consuming and bedside testing is not yet possible.

Furthermore, detection of miRNAs compared with the detection of protein-based biomarkers still lacks standardized methods and automatized work flows as well as clear recommendation as to what body fluid to use for what type of miRNA.

Normalization

An important technical aspect concerning miRNA quantification lies in the need for standardized and reproducible isolation and detection protocols as well as uniform means of analyses and normalization in order to efficiently generate robust and reproducible results. Different methods of normalization have been described to have an effect on miRNA quantification (140). There are no standardized control transcripts for normalization, which resulted in the generation of various normalization strategies (141). A widely used method is based on *C elegans* miR-39 spike in control as a reference miRNA. On the other hand standardization to the mean of all measured miRNAs as well as to individual miRNAs that are detectable at similar levels in all study subjects is highly recommended (142).

Comparing serum and plasma: differing miRNA levels

While some authors reported similar levels of miRNAs in

serum and plasma (7,25), inconsistencies between miRNA levels in both materials have also been found (143). Notable is the observation that especially thrombocyte-associated miRNAs in plasma are found in much higher concentrations than in serum (73). Since especially in the field of miRNA-based diagnostics of CAD thrombocyte-derived miRNAs such as miR-126, miR-223 and miR-197 seem to play a significant role in the prognosis of future cardiovascular events (27), standardized methods are needed for future clinical miRNA quantification studies.

Parameters influencing miRNAs levels

Former studies have proven that heparin administered to patients prior to blood sampling interferes with results in miRNA quantification (144,145). Therefore, high caution is needed when selecting patients for *in vivo* studies of miRNA quantification with respect to heparin administration prior to blood sampling.

Quantification of miRNA levels altered in CAD might also be influenced by the intake of medication such as statins and ACE inhibitors (102).

These findings emphasize the importance to quantify the drug- and metabolite-based influence on miRNAs in the clinical setting. At the same time the inconsistency of the data reflects the necessity of further studies evaluating pathways of how miRNA levels are influenced in circulating blood.

Additionally, it needs to be considered, that levels of biomarkers can also be influenced by the speed of their elimination. Gidlöf *et al.* found cardiac miRNA levels strongly correlating with renal function indicating that the renal function might also influence the plasma levels of miRNAs (84).

Future steps

Despite a tremendous increase of interest in miRNAs for cardiovascular disease diagnosis and prognosis, we are only just scratching the surface of its complexity. To fully elucidate the potential of miRNAs robust sample processing as well as advances in technology and analysis methods are required.

Discovery of novel miRNAs and confirmation of reported results should be a component of large research networks, involving industry and experts in distinct fields such as molecular biology, analytical chemistry, bioinformatics, clinical-trial design, epidemiology, statistics as well as health-care economics (146). Several

collaborative initiatives have emerged in recent years to orchestrate biomarker research efforts. One is the EU funded BiomarCaRE consortium (Biomarker for Cardiovascular Risk Assessment in Europe), a European collaborative research project including over 300,000 participants from 13 European countries in clinical as well as diseased cohorts (147). Amongst other biomarkers miRNAs are evaluated with respect to their predictive value for incident CAD and MI. Quantification of circulating miRNAs on such a large scale has never been performed before and represents the chance to possibly validate findings of the numerous single miRNA studies that have been performed so far.

New players in the field: long non-coding RNAs (lncRNAs)

Besides miRNAs lncRNAs have increasingly attracted attention in the field of gene regulation recently (148). lncRNAs consist of >200 nucleotides and are involved in regulatory mechanisms of gene expression (149). Circulating lncRNAs are being evaluated as biomarkers for cardiovascular disease. First studies revealed differential levels of lncRNAs in blood cells of 414 patients with acute MI (150), suggesting the involvement of lncRNA in pathophysiological conditions of acute MI and their potential future application as circulating cardiovascular biomarkers.

Summary

Numerous studies reported miRNAs as promising new diagnostic and prognostic biomarkers in the field of cardiovascular diseases. The application of circulating miRNAs as biomarkers represents a potential additional module in disease diagnosis and prognosis complimentary to established protein-based biomarkers. *In vitro* testing and animal models have depicted pathophysiological pathways and authors have developed miRNA-based methods to interfere with disease progression and complications. The use of miR-mimics and antagomirs in the cardiovascular field has not yet found its way into clinical trials but promising study results reflect potential future applications of miRNA therapeutics in this field.

The complex regulatory mechanisms involved in miRNA and gene expression have not yet been completely understood. At the same time technical aspects of detecting and quantifying miRNAs in body fluids including RNA

normalization and biomaterial to be used need to be standardized as a basis for reproducible protocols.

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