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Small RNA Biomarkers Come of Age*

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MicroRNAs (miRNAs) are endogenous, 21- to 23-nucleotide-long RNA molecules that post-transcriptionally regulate target genes. miRNAs interact specifically with certain mRNAs by repressing their translation or inducing their degradation (1). Current estimates are that miRNAs thereby regulate the levels of the majority of mammalian proteins (2). Research in recent years has assigned miRNAs a key regulatory role in various physiological processes. Also, a growing number of miRNAs has been implicated in the pathogenesis of several diseases, including cardiac disease (3). With respect to the latter, miRNAs have been implicated in cardiac hypertrophy (4,5), fibrosis (6), and vascular disease (7). Intriguingly, miRNAs also appear to represent valid therapeutic targets, because modulation of their expression in vivo (for example, with antisense RNA molecules) has been shown to effectively modulate cardiovascular disease in various animal models (4,6,8–10). Most recently, miRNAs have received much attention regarding their suitability as biomarkers for disease. Following pioneering work from the cancer field (11,12), also several cardiovascular studies have found marked deregulation of miRNAs in various types of clinical specimens (for review, see ref. 13).

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What is the advantage of measuring levels of circulating miRNAs, given the wealth of information on protein biomarkers? There are several reasons. 1) As nucleic acids, miRNAs can be both amplified and detected with high sensitivity and specificity. 2) miRNA arrays and quantitative PCR (qPCR) methodology allow the quantification of many miRNAs in a single experiment. There is evidence that the combined analysis of miRNAs and their coexpression pattern (miRNA networks) enhances the predictive power. 3) miRNAs are relatively stable over time in human blood and appear to be protected from degradation through various mechanisms.

On the contrary, the quantitative analysis of miRNAs in material such as blood and urine has certain disadvantages: 1) The concentrations of most circulating miRNAs are typically very low, which makes reliable quantitation and normalization a challenge with existing technology. Also, there is no consensus for normalization controls. 2) Current qPCR and microarray technologies are still time-consuming (several hours) compared with some protein-based biomarker tests such as troponin or C-reactive protein, which offer results within minutes. 3) For now, the added value of miRNA-based biomarkers remains to be established by more rigorous testing and comparison to established biomarkers. Despite these hurdles, several laboratories have already obtained profiles of circulating miRNAs in cardiovascular disease and explored their biomarker potential (see Table 1 for an overview [14–24]). What is immediately apparent, are certain inconsistencies between studies, where the same or highly similar settings have been studied. This can be partially attributed to the current immaturity of the field, which still includes technical issues such as variability of RNA extraction protocols, different means of nucleic acid detection and normalization procedures. However, many studies are simply underpowered (too low numbers of patients) and/or do not use appropriate controls matched for potentially confounding factors such as age, sex, medication, and comorbidities. Also, there is little comparison of miRNAs to reference biomarkers.

It is against this background that Zampetaki et al. (24) report in this issue of the *Journal* the first prospective cohort on the determination of circulating miRNAs in cardiovascular disease. Their study design should set the bar markedly higher for future studies on miRNAs as biomarkers for cardiovascular disease. As a continuation of a similar study on type 2 diabetes (25), Zampetaki et al. (24) have now quantified 19 circulating miRNAs in 820 participants of a population-based study in Bruneck, Italy. Among these, 3 miRNAs (namely, miR-126, miR-197, and miR-223) are reported to be significantly associated with the risk of future myocardial infarction. This association resulted from multivariable statistical analysis (i.e., multiple potentially confounding parameters were taken into account). Importantly, the authors report that the combination of these 3 miRNAs improves the Framingham Risk Score for hard coronary heart disease, which is based

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Table 1 Selected Studies of Plasma/Serum miRNAs in CHD

Disease (n)	Controls (n)	miRs With Altered Serum/ Plasma Concentration in Disease	Comparison With Established Biomarker	Ref. #
Case-control studies, cross-sectional				
CHD (36)	Healthy (17)	Down: miR-17, -92a, -126, -145, -155, -199a	—	14
CHD (31)	Non-CHD (14)	Up: miR-133a		
		Down: miR-17, -92a, -126, -145, -155		
AMI (19)	CHD non-AMI (31)	Up: miR-92, -126, -133a, -208a, -499	Correlation of hsTnT with miR-133a, -499	15
	No CHD (7)			
AMI (33)	Healthy (30) CV disease other (17) CHD non-AMI (16)	Up: miR-1, -133a, -208a, -499	TnT	16
AMI (33)	Healthy (17)	Up: miR-1, -133a, -133b, -499-5p	—	17
		Down: miR-122, -375		
AMI (9) UAP (5)	Healthy (10)	Up: miR-499	—	18
AMI (93)	Healthy (66)	Up: miR-1	QRS duration, no correlation with TnI, CK-MB ROC analysis	19
AMI (31)	Healthy (20)	Up: miR-1	CK-MB	20
ACS (29)	Non-ACS (42)	Up. miR-1, -133a	Correlation with CPK, CK-MB and cTnT ROC analysis	21
AMI (32)	Chest pain, non-AMI (36)	Up: miR-133a, -208b, -499	Correlation of miR-208b and -499 with TnT	22
		Down: miR-223		
			ROC analysis	
ACS cohort STEMI (196), NSTEMI (131)	UAP (117)	Up: miR-1, -133a, -208b	Correlation of hsTnT with 1, miR-133a, -133b, -208b	23

Cohort (n)	Incident Event	miRs With Predictive Value for Incident Event	Predictive Value in Addition to Established Biomarker	Ref. #
Prospective studies				
ACS cohort (444)	Death (34)	Up: miR-133a, -208b	None in addition to hsTnT	23
Bruneck cohort (820)	AMI (47)	Up: miR-126	Predictive value in addition to Framingham Risk Score	24
		Down: miR-197, -223		

ACS = acute coronary syndrome(s); AMI = acute myocardial infarction; CHD = coronary heart disease; CK-MB = creatine kinase myocardial band; CPK = creatine phosphokinase; cTnT = cardiac troponin T; CV = cardiovascular disease; hsTnT = high-sensitivity troponin T; ROC = receiver-operating characteristic; NSTEMI = non–ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction; TnI = troponin I; TnT = troponin T; UAP = unstable angina pectoris.

on the established risk factors age, sex, total and high-density lipoprotein cholesterol, blood pressure, and smoking status (26). The improvement of risk classification amounted to nearly 15%, which is remarkable and exceeded the impact of alternative candidate biomarkers such as C-reactive protein.

A second part of this work aims to provoke changes in plasma miRNA levels through a controlled intervention in healthy volunteers. The intervention consisted of 30 min of thigh cuff inflation resulting in leg ischemia and repetitive sampling post-injury. Although this model cannot mimic the situation of incident MI in the Bruneck cohort, the results support coregulation of these 3 miRNAs because they were all up-regulated in plasma alongside 2 additional miRs, miR-21, and miR-24.

What are the principal merits of this study? 1) This study provides for the first time an miRNA-based biomarker signature for cardiovascular disease that adds predictive power to an established standard, in this case, the Framingham Risk Score for hard coronary heart disease. 2) The study is performed in a prospective, population-based cohort. The results of this type of studies can be expected to be more robust than those of studies using healthy controls. 3) The intervention part in healthy volunteers provides interesting clues with regard to the potential origin of some biomarker miRNAs. With regard to the latter, the concomitant increase in miR-126, miR-197, and miR-223 during thigh cuff ischemia raises several interesting questions: From where do these miRNAs originate? How are they transported and stabilized in the blood? Are they taken up by target cells? If yes, by what mechanisms and by what cell type? Are the concentrations in plasma and in potential target cells sufficiently high to exert biological effects? Most of these