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MicroRNAs: From Basic Mechanisms to Clinical Application in Cardiovascular Medicine

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MicroRNAs Within the Continuum of Postgenomics Biomarker Discovery

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Abstract—The postgenomic shift in paradigm from reductionism to systems-wide network inference has increased recognition that cardiovascular diseases are not simply determined by the genome but arise from an interaction and dynamic dysregulation of gene regulatory networks, proteins, and metabolic alterations. The advent of postgenomic technologies promises to interrogate these complex pathophysiological perturbations by applying concepts of systemic relationships to biomarker discovery. A multibiomarker panel consisting of biomarkers capturing different levels of information (eg, microRNAs to assess endothelial and platelet activation, molecular lipid species to profile metabolic status, and proteolytic degradation products to assess vascular integrity) could outperform inflammatory biomarkers without vascular specificity in their ability of predicting cardiovascular risk. As atherosclerosis develops over decades, different biomarkers may be required for different stages of disease. Thus far, there is no simple blood test to directly assess the health of blood vessels or identify vulnerable patients. We discuss strategies for biomarker discovery using post genomics technologies, with a particular focus on circulating microRNAs. The aim is to reveal distinctive cardiovascular phenotypes and identify biomarker signatures that complement the Framingham risk scores in clinical decision-making and in a stratified medicine approach for early preventive treatment of disease. **(*Arterioscler Thromb Vasc Biol*. 2013;33:206-214.)**

Key Words: biomarker ■ lipids ■ metabolomics ■ microRNAs ■ proteomics

Is There a Need for New Cardiovascular Biomarkers?

Discovery of novel biomarkers and improvement of risk prediction algorithms will be a key to fulfill the promise of personalized medicine. Although genuine personalized treatment is probably an unrealistic expectation within the budget constraints of current health systems, a stratified medicine approach to identify individuals at high risk may help to allocate available resources most efficiently in a setting with a rising epidemic of cardiovascular disease (CVD) fueled by obesity, hypertension, and diabetes mellitus and by an aging population. Besides, biomarkers may improve patient motivation; by facilitating risk communication and compliance to lifestyle changes and therapies.

The search for cardiovascular biomarkers dates back to the mid 1960s when creatine kinase and its cardiospecific isoform creatine kinase-MB were established as indicators of acute myocardial damage.[1] About a decade later, the Framingham Study pioneered the quest for long-term cardiovascular risk prediction. Framingham risk scores,[2] as well as others, such as Prospective Cardiovascular Münster (PROCAM) study, Systemic Coronary Risk Evaluation (SCORE), and Reynolds,[3] are widely accepted and implemented tools in clinical decision-making. These scores inform treatment decisions, but they leave ample room for improvement. Currently, the majority of cardiovascular risk is not explained by traditional risk factors. In fact, most events occur in patients with an average risk score who are erroneously deemed to be at intermediate or low risk because they have no or only 1 of the cardiovascular risk factors.[4] In contrast, many high-risk individuals do not experience a cardiovascular event even in the long term.[5] Thus, the renewed interest in biomarkers is warranted, and the recent advances in postgenomic technologies offer unprecedented opportunities for biomarker discovery.[6]

How Well Do Current Cardiovascular Risk Factors Predict Risk?

Up to now, biomarkers linked to systemic and vascular inflammation, oxidative stress, or vascular calcification received most of the attention. In the latest (2010) release of the American College of Cardiology Foundation/American Heart Association guidelines for cardiovascular risk assessment in asymptomatic individuals,[3] a few of these biomarkers (C-reactive protein, hemoglobin A1C, urinary albumin excretion, and lipoprotein-associated phospholipase A2) were given a class IIa or IIb recommendation for clinical use in particular subgroups. Others like natriuretic peptides[7] or oxidized phospholipids on

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apoB-100–containing lipoproteins[8,9] show promise and are currently being tested to establish their additive predictive value.

CVD is inherently so complex that a single biomarker is unlikely to capture the individual predisposition to develop CVD or herald its sequelae. Also, it is challenging to improve the established cardiovascular risk scores by measuring new biomarkers and achieve meaningful incremental values over and above traditional risk factors. For example, C-reactive protein improves cardiovascular risk prediction in the general population, but its incremental predictive value is somehow limited because of its strong correlation with other risk factors, such as body mass index and smoking.[10] Conventional risk scores already combine different levels of information (age, sex, family history, smoking status, hypercholesterolemia, hypertension, and diabetes mellitus) by relatively inexpensive measurements that can be readily performed in the clinic. To recapitulate this multirisk factor strategy, advanced statistical and bioinformatics methods should be used to identify biomarker signatures that provide complementary information (ie, by determining the response of an individual to a given burden of cardiovascular risk factors).

Clinical Use of Genetic Testing for Cardiovascular Risk Prediction?

Although heredity encompasses an important aspect in the development of CVD and genome-wide association studies have unraveled intriguing susceptibility loci, the advance in cardiovascular risk prediction offered by genetic markers is modest at best.[11] As a trait with complex genetic causes, CVD does not follow readily predictable patterns of inheritance but results from variation within multiple genes and their interaction with behavioral and environmental factors. Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) for cardiovascular risk, but the risk alleles are common and have small effects (odds ratios of 1.1–1.3). Combining modest-risk genotypes, genes involved in lipid metabolism, endothelial function, and clotting did result in a significant 12% net reclassification improvement compared with Framingham risk score, by using 13 meta-analysis proven candidate gene SNPs and adding 7 genome-wide association SNPs.[12] Nonetheless, the American College of Cardiology Foundation/American Heart Association guidelines[3] opt for a thorough assessment of CVD family history but recommend against genetic testing for SNPs in CVD susceptibility genes. At present, it seems unlikely that genetic testing for cardiovascular risk will have a wider clinical use. The genotype may influence cardiovascular risk, but this risk is mediated through its impact on trait. Most genotypes will not predict risk over and above measures of cognate trait. Also, there is an urgent need to translate the results from genome-wide association studies into mechanistic insights. What is missing are those carved paths of linking SNPs to cell signal transduction and metabolism to promote our understanding of pathogenetic mechanisms.

Post Genomics Technologies: Beyond Individual Biomarkers

The assessment of individual biomarkers has provided much insight, but complimentary approaches are being pursued to advance CVD risk prediction:

1. The development of multistage risk scores or targeted scores for patient subgroups diverging from the current strategy of "one prediction tool fits all."
2. The consideration of measures of preclinical disease status similar to cancer medicine. Coronary artery calcium, carotid artery intima-media thickness,[13] and plaques are assumed to reflect life-time exposure to known and unknown risk factors and their interaction with an individual's genetic background.
3. To go beyond genomics and interrogate the transcriptome, proteome, metabolome, and lipidome and move from solitary serum biomarkers, most of which are not specific to the vasculature, to biomarker signatures, and to biomarker networks.

The genetic specification of a human being, once assumed to be of almost limitless complexity, consists of just 20 000 protein-coding genes. Only ≈1.5% of the human genome codes for proteins. Yet, >80% of the human genome is actively transcribed.[14] These noncoding regions of the human genome have previously been termed junk DNA. It is now clear that biological complexity is determined not by the number of protein-coding genes (*C. elegans* has a similar number of protein-coding genes as humans, rice plants have twice as many), but by the regulation of these gene products. This shift in perspective quickly led to the creation of postgenomic sciences: transcriptomics for RNAs, proteomics for proteins, and metabolomics for small molecules. The genetic make-up of an individual may predispose to CVD but neither can it predict its onset nor progression. Biomarkers originating from postgenomic technologies are closer to the CVD phenotype than SNPs and provide information on epigenetic regulation, cell activation, tissue repair, and metabolic processes within the vasculature that may not be captured by standard risk factors. Given the recent advances in assessment methods (postgenomics technologies) and biostatistics (methods capable of handling extensive amounts of data with high dimensionality and collinearity, network inference algorithms), this new phase in biomarker discovery and vascular risk prediction offers the realistic hope for improving patient stratification and clinical management.

Circulating MicroRNAs

MicroRNAs (miRNA) are short segments of RNA that are not translated into proteins, but function as posttranscriptional regulators of gene expression and have been implicated in CVD. Seminal studies by Mitchell et al[15] have revealed the presence of endogenous miRNAs in the circulation that are not cell-associated. Unlike messenger RNAs, miRNAs are stable in blood. In the circulation, miRNAs are protected from RNAse activity by microvesicles[16,17] (exosomes, microparticles, and apoptotic bodies), RNA-binding proteins (eg, Ago2 complexes),[18] or lipoproteins (low-density lipoproteins and high-density lipoprotein).[19] Thus, besides their classical role as a delivery vehicle for cholesterol, lipoproteins may also act as a carrier or depot for endogenous miRNAs and facilitate their transport and delivery to recipient cells.[19] The existence of a miRNA pool within the circulation is an exciting new aspect of current biology and attracting considerable attention.[20] miRNAs may offer distinct advantages over other biomarkers[21]:

1. Unlike messenger RNAs, miRNAs are stable in blood. Circulating cells are probably the major contributor to the extracellular miRNA pool. Acute organ damage can lead to an increase of tissue-specific miRNAs (eg, cardiac miR-1 becomes detectable in the circulation after acute myocardial infarction [AMI]),[22] but it is not a foregone conclusion that changes in tissue miRNAs will give rise to a corresponding change in circulating levels.
2. As nucleic acids, miRNAs can be both amplified and detected with high sensitivity and specificity. Unlike protein-based biomarkers that tend to be measured individually, real-time polymerase chain reaction methodology allows the multiplexing of several miRNAs in a single experiment.
3. Because most circulating miRNAs are highly correlated, global patterns of expression should be studied by representing miRNA data as coexpression networks. Apart from their relative levels, it is the interaction and connectivity of a miRNA within the miRNA network that defines disease-specific signatures and helps to unravel cell-type–specific expression patterns.[23] For many protein biomarkers, the cellular origin remains uncertain. For example, both endothelial cells as well as platelets secrete von Willebrand factor. Measuring its circulating levels does not reveal how much of von Willebrand factor is endothelial- or platelet-derived. The same limitation applies to most cytokines and chemokines that are detected in the circulation.

miRNAs as Novel Biomarkers for Cardiovascular Risk Prediction

To identify changes in circulating miRNAs that might precede subsequent cardiovascular events, we measured miRNAs in the Bruneck study.[24,25] This study stands out with its prospective design and a population cohort of 820 individuals. Most studies published on miRNAs to date are small case-control studies comparing patients with manifest disease and healthy controls (Table).[24–43] Evidence based on case-control comparisons has to be interpreted with caution because miRNAs are measured after disease onset and a temporal relationship cannot be established (reverse causality). Also, there is a need to standardize normalization procedures and introduce minimum requirements for statistical analysis.[44] Prospective population-based studies comprise a wide range of individuals representative of the general community, and samples are taken before the onset of disease offering a better opportunity to identify miRNA signatures with specificity for CVD. Because it is currently unclear how cardiovascular risk factors impact on RNA biomarkers, our aim was to first assess the response of miRNAs to cardiovascular risk factors before identifying miRNA signatures of risk of AMI. The principal findings of these studies are as follows:[21]

1. Among all cardiovascular risk factors tested (hypercholesterolemia, type II diabetes mellitus, smoking, and hypertension), type II diabetes mellitus had the most pronounced effect on circulating miRNA profiles.[24]
2. A miRNA-based biomarker signature comprising just 3 (miR-126, miR-223, and miR-197) of 19 miRNAs (Figure 1A) added information to an established standard, the Framingham Risk Score for Hard Coronary Heart Disease.[25] The integrated discrimination improvement, Akaike information criterion, and net reclassification index were used to assess to what extent adding information on miRNAs stratifies patients to risk categories that better reflected their disease outcome (Figure 1B).
3. The associations for these miRNAs and outcome events (with an expected 1.5–2-fold increase in risk per 1 SD change) were stronger than for C-reactive protein and other risk factors (≈1.3-fold increase in risk per 1 SD unit). Adjustment for known cardiovascular risk factors had only marginal effects on the risk estimates obtained (values of hazard ratios were changing by <5%).[25]
4. The population study was accompanied by an intervention study in healthy individuals to determine their cellular origin. On the basis of expression profiles after limb ischemia-reperfusion injury, miR-126, miR-197, and miR-223 were part of 1 cluster that also included miR-21 and miR-24. All these miRNAs are highly expressed in platelets and platelet microparticles.[25] This is, to our knowledge, the first time that the contribution of a specific cell type to circulating miRNAs has been defined by a controlled intervention.

The diagnostic and prognostic potential of circulating miRNAs requires confirmation and cross-validation in follow-up studies as well as independent cohorts. miRNA measurements before and after cardiovascular events during observation will be the ideal setting to define which miRNA pattern antedate the event and which develop as a response of disease manifestation (reverse causality). In addition, measurements are currently performed in plasma and serum. No study has as yet addressed the compartmentalization of circulating miRNAs in the context of cardiovascular risk prediction.

Cellular Origin of Circulating miRNAs Associated With Cardiovascular Risk

Atherothrombosis is a key event in AMI and constitutes an important mechanism for advanced but not early progression of atherosclerosis. Advanced stages of atherosclerosis do not rely on traditional risk factors and are not adequately captured by measurements of intima-media thickness. In the Bruneck study, we observed a stronger correlation of miRNAs with AMI rather than with surrogate measures of early atherosclerosis. This is in agreement with our finding in the interventional study that at least 2 of the 3 miRNAs (miR-223 and miR-197) constituting the signature for AMI are platelet-derived. Although miR-126 is highly enriched in endothelial cells, miR-126 is also present in platelet, albeit at much lower levels. Nonetheless, platelets are the second most abundant cell type in the circulation, and the shedding of platelet microparticles in plasma or serum is a major contributor to circulating miR-126 levels. At least in healthy volunteers, there is a good correlation between miR-126 plasma levels and platelet microparticles.[25] By measuring additional miRNAs, such as miR-223, that are abundant in platelets but show low expression in endothelial cells, one can refine the endothelial contribution to the total miR-126 content in the circulation (Figure 2). The present findings extend our previous observations in patients with diabetes mellitus and raise the possibility that the observed loss of several miRNAs, including miR-126,

Table. Summary of Clinical Studies on miRNA Biomarkers

First Author[reference]	Description of Study Population	Study Size	No. of miRNAs Measured	Normalization	Statistical Methods			Additional Correlations
					Statistical Adjustment*	Methods Used to Account for Collinearity Between miRNAs	Assessment of Value in Risk Prediction	
Prospective studies/general population								
Zampetaki et al[24]	General population → DM2 over 10 years	80 vs 80, 19 vs 19†	13	RNU6B, miR-454	++	Network analysis	…	Impaired glucose tolerance
Zampetaki et al[25]	General population → AMI over 10 years	820	19	U6, Ct average	++	Network analysis, L1 penalization, best subset (AIC)	C-index, NRI, IDI	Thigh cuff ischemia/ reperfusion
Prospective studies/patients with preexisting CVD								
Widera et al[26]	ACS patients → all-cause mortality over 6 months	444	6	Cel-miR-54	++	…	AUC	hs-cTnT
Eitel et al[27]	ACS patients → MACE over 6 months	216	1	Cel-miR-39	++	Only 1 miRNA tested	…	Cardiac MRI
Case-control studies/acute coronary syndromes (ACS)								
Ai et al[28]	AMI patients vs hospital controls	93 vs 66	2	U6	o	…	AUC	cTnI, CK-MB
Wang et al[29]	AMI patients vs non-AMI patients vs healthy controls	33 vs 33 vs 30	6	Cel-miR-39	o	…	AUC	cTnT
D'Alessandra et al[30]	STEMI patients vs healthy controls	33 vs 17	6	miR-17-5p	o	…	…	…
Adachi et al[31]	ACS vs HF vs controls	14 vs 15 vs 10	1	Internal reference small RNA	o	Only 1 miRNA tested	…	…
Cheng et al[32]	AMI vs controls	31 vs 20	1	?	+	Only 1 miRNA tested	…	CK-MB
Kuwabara et al[33]	ACS vs non-ACS patients	29 vs 42	2	?	o	…	AUC	cTnT
Oerlemans et al[34]	ACS vs non-ACS patients	106 vs 226	5	RNU6	++	…	AUC	hs-cTnT
Long et al[35]	AMI vs healthy controls	17 vs 25	2	RNU6	o	…	AUC	cTnI
Devaux et al[36]	NSTEMI vs STEMI vs healthy controls	113 vs 397 vs 87	2	3 Cel-miRNAs	o	…	AUC, NRI	CK-MB, cTnT, hs-cTnT
Case-control studies/coronary artery disease (CAD)								
Fichtlscherer et al[37]	CAD patients vs healthy controls	67 vs 31	8	Cel-miR-39	o	…	…	Baseline characteristics
Gao et al[38]	Hyperlipidemic patients with and without CAD vs normolipidemic controls	255 vs 100	4	Cel-miR-39	++	…	…	Total cholesterol, triglycerides, LDL, HDL
Sun et al[39]	CAD vs non-CAD patients	31 vs 36	1	miR-16	o	Only 1 miRNA tested	…	LDL
Case-control studies/heart failure								
Tijsen AJ et al[40]	HF vs non HF vs healthy controls	30 vs 20 vs 39	16	miR-1249	+	…	AUC	BNP and ejection fraction
Goren et al[41]	HF patients vs controls	30 vs 30	186;4 validated	Ct average	o	…	AUC	BNP

(*Continued*)

Table. Continued

First Author[reference]	Description of Study Population	Study Size	No. of miRNAs Measured	Normalization	Statistical Methods			Additional Correlations
					Statistical Adjustment*	Methods Used to Account for Collinearity Between miRNAs	Assessment of Value in Risk Prediction	
Corsten et al[42]	AMI vs control patients with chest pain; acute viral myocarditis vs postmyocarditis vs controls; diastolic dysfunction vs hypertensive vs normotensive patients; acute HF vs controls	32 vs 36; 14 vs 20 vs 20; 39 vs 20 vs 20; 34 vs 33	9	3 Cel-miRNAs	o	…	AUC	BNP, creatine phosphokinase, cTnT, other clinical parameters
Olivieri et al[43]	NSTEMI vs HF vs control	92 vs 81 vs 99	6	miR-17, Cel-miR-39	o	…	AUC	hs-cTnT

*Degree of adjustment: **o** indicates unadjusted; **+** minimally adjusted (typically adjusted for age and sex only); **++** additional adjustment for other cardiovascular risk factors; ?, not specified; and ..., not determined.

ACS indicates acute coronary syndromes; AIC, Akaike information criterion; AMI, acute myocardial infarction; AUC, area under the receiver operating characteristic curve; BNP, brain natriuretic peptide; CAD, coronary artery disease; Cel, *C. elegans*; cTnI, cardiac troponin I; cTnT, cardiac troponin T; hs-cTnT, high sensitivity cTnT; CK-MB, creatine kinase-MB; DM2, type 2 diabetes mellitus; EF, ejection fraction; HDL; high-density lipoproteins; HF, heart failure; IDI, integrated discrimination improvement; LDL, low-density lipoprotein; MACE, major adverse cardiovascular events (defined as a composite of death, reinfarction and new congestive heart failure); MRI, magnetic resonance imaging; NRI, net reclassification index; NSTEMI, non-ST elevation myocardial infarction; and STEMI, ST-elevation myocardial infarction.

†The study is based on a case-control comparison (n=80 per group) nested in the prospective Bruneck studies and includes a comparison of incident diabetes mellitus plus matched controls (n=19 per group).

miR-197, miR-223, miR-24 and miR-21, may reflect abnormal platelet function in diabetic patients.[24] Also, previous comparisons of circulating miRNAs between healthy volunteers and patients with CVD are likely to be confounded by medication, in particular antiplatelet therapy.

Mechanistic Links Between Circulating miRNAs and Cardiovascular Risk

To expand mechanistic insights between miRNAs and cardiovascular risk, further downstream analysis will include identifying putative protein targets for these miRNAs. The 3 miRNAs implicated in cardiovascular risk have a mechanistic underpinning in endothelial damage and platelet dysfunction. Currently, most studies rely on bioinformatic algorithms or transcript analysis for the identification of miRNA targets. These algorithms are based on an incomplete understanding of miRNA-mRNA seed pairing and evolutionary conservation of miRNAs. They typically predict hundreds to thousands of target genes for 1 miRNA, but with limited overlap, and even the most sensitive programs fail to identify known targets. Importantly, cell type context is not taken into consideration. A considerable proportion of miRNAs act as translational inhibitors, and many effects can only be observed at the protein level and not at the mRNA level. The use of proteomics methods will be essential for a more comprehensive understanding of miRNA-mediated regulation of gene expression.[45] Similarly, metabolomic techniques should be used to more thoroughly investigate the correlation of miRNAs with clusters of metabolites.[46]

Lipidomics: Molecular Lipid Profiling for Cardiovascular Risk Prediction

Our knowledge about lipid biomarkers is mostly related to the classes of lipids rather than to single lipid species within the class. Biomarker studies investigating lipids focused on triglycerides, high-density lipoprotein, low-density lipoproteins, cholesterol, and their derivatives, which have been shown to be involved in the pathophysiology and progression of the disease. With regard to cardiovascular risk prediction, no detailed comparison of individual lipid species across these different classes has been performed till date. Targeting specific lipid species that are most atherogenic could result in a better classification of cardiovascular risk than relying on conventional lipid measurements.[47]

We have recently used a shotgun lipidomics platform to provide a comparative lipidomics analysis of human endarterectomy samples.[48] Shotgun lipidomics as described by Han and Gross[49] uses the different scan options of a triple-quadruple mass spectrometer to resolve isobaric lipids from different lipid subclasses and to detect even minor components, which would otherwise be masked by the presence of abundant lipid species. First a survey scan in positive and negative mode is acquired. Then, different product ion and neutral loss scans, characteristic for the different lipid classes, are used for the unambiguous identification of certain lipid classes by their product ions. This mass spectrometer-based intrasource separation technique allows the identification and quantification of hundreds of individual lipid species in a complex biological sample. A liquid extraction-based surface sampling device was adapted for the analysis of plaque lipids directly from tissue sections. Both methods, liquid extraction surface analysis from tissue sections and conventional analysis by preparing Folch extracts, showed similar results in terms of detected signals and their intensity. In total, 150 lipid species from 9 different classes were identified, of which 24 were detected in endarterectomy specimens only but not in healthy arteries.[48] For

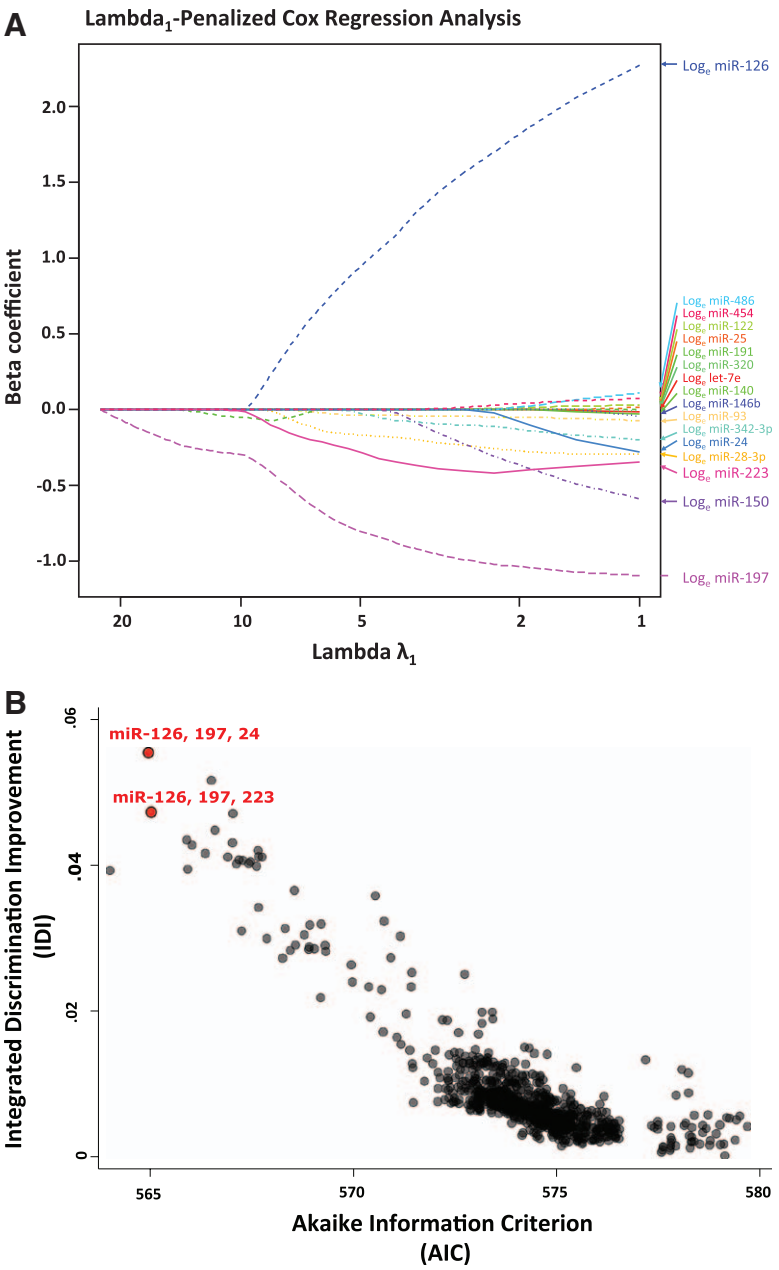

Figure 1. miRNA signature for incident myocardial infarction. **A**, λ_1-penalized Cox regression analysis (least absolute shrinkage and selection operator method). The graph shows Cox regression coefficients of miRNAs (*y* axis) for different levels of penalization (the tuning parameter λ_1 escalates >20). Variables withstanding shrinkage up to high λ_1 values are those most relevant for disease prediction (reproduced from Ref. 25). **B**, All combinations of eligible miRNAs were computed and compared according to the models' Akaike information criterion (AIC) that is based on the maximized log-likelihood and imposes a penalty for increasing the number of parameters in the model. Lower values of AIC indicate the preferred model which is the one with the fewest parameters still providing adequate fit (tradeoff between accuracy and complexity). The net reclassification improvement (NRI) formula was used to assess the extent to which adding information on miRNAs reassigns participants to risk categories that better reflect their CVD outcome (10-year risk categories <10%, 10%–20%, and >20%). Approximate 95% CIs for the NRI were calculated by using the same variance terms as in the test of significance. Unlike the NRI, the calculation of the integrated discrimination improvement (IDI) does not rely on arbitrarily chosen cutoffs for 10-year risk categories. The combinations of all miRNAs were plotted based on their improvement of the AIC and the IDI to demonstrate that our preferred combinations of miRNAs (miR-126, miR-197, miR-223/miR-24) are the best among all miRNA combinations tested. Note that miR-223 and miR-24 are highly correlated and thus interchangeable.

quantitation, authentic standards of the different lipid classes were spiked in Folch extracts. Several lipids were reduced in regions with evidence of rupture, and one could envisage that leakage of these plaque-related lipid products into the circulation may constitute a better marker for plaque burden and vulnerability than total cholesterol levels and plasma lipoproteins. For example, Meikle et al[50] used a target-focused analysis of plasma lipids to highlight the potential importance of measuring distinct lipid species rather than lipid classes in patients with stable and unstable coronary artery disease.

Proteomics: Assessing Vascular Integrity by Monitoring Proteolysis

With regard to biomarker discovery, proteomics has overpromised but underdelivered. The cardiovascular field is no exception.[51] The plasma proteome is the most complex proteome of the human body. Current mass spectrometry techniques offer 4 to 5 orders of magnitude of linear dynamic range. Yet, the dynamic range of biomarkers routinely measured in the clinics spans >10 orders of magnitude.[52] Undersampling results in an overrepresentation of classical plasma proteins in proteomic data sets. Unlike tissue leakage products, such as cardiac troponins, changes in abundant plasma proteins frequently lack specificity for disease. An alternative strategy is the application of proteomics directly to the diseased tissue, where the potential biomarkers are less dilute.[53] Once biomarker candidates have been identified, they can be quantified in the circulation by conventional techniques that are less affected by the presence of high abundant plasma proteins.

Although others have focused on circulating cells to identify cardiovascular biomarkers,[54,55] we have developed a proteomics method to analyze the composition of the

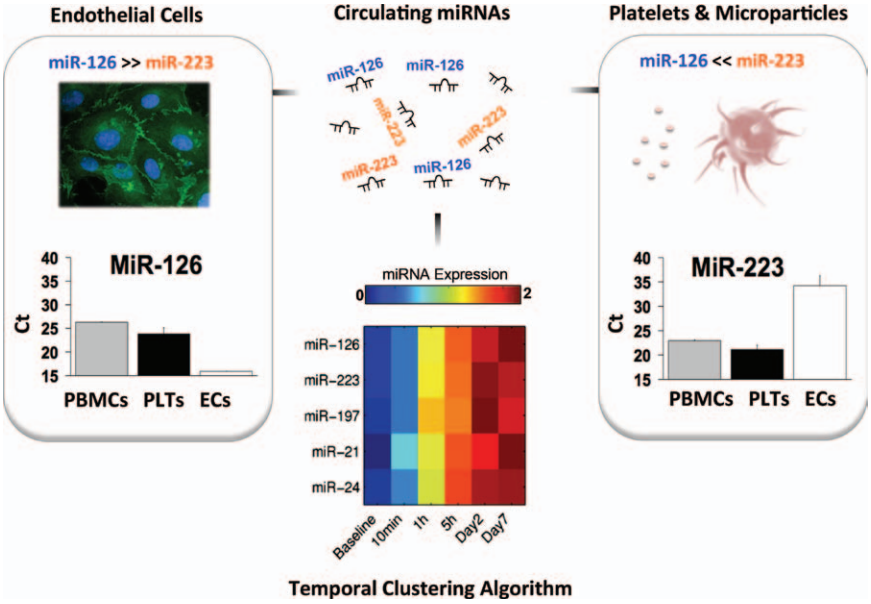

Figure 2. Endothelial and platelet contribution to miR-126 levels. MiR-126 is more abundant in endothelial cells than in platelets. The opposite is the case for miR-223. In healthy volunteers, we observed a coordinated increase of miR-126 and miR-223 after ischemia/reperfusion injury by thigh cuff inflation, suggesting a common platelet origin. This association is highlighted by computational analysis using the temporal clustering by an affinity propagation algorithm based on average miRNA expression at baseline and over time (10 min, 1 h, 5 h, 2 d, 7 d). Ct indicates cycle threshold; PMBCs, peripheral blood mononuclear cells; PLT, platelets; and EC, endothelial cells.

cardiovascular extracellular matrix.[56,57] By using a decellularization step, we overcome differences in cellular heterogeneity. Tissue heterogeneity is a major concern for any omics technology. Most clinical samples represent an endstage of human disease. In whole tissue, changes between controls and disease may reflect changes in the proportion of cell populations (ie, in inflammatory infiltration) rather than differential expression within a resident cell type. This problem is exaggerated with ever more sensitive techniques, such as next generation sequencing. Without information on cell composition, omics experiments may become an expensive substitution for histology. Besides, transcriptomics provides only a snapshot of gene expression at 1 time point; mRNAs are not informative about the events that occurred during the disease process or proteolytic activity. Instead, the extracellular matrix provides a read-out of vascular integrity and the inflicted damage during disease progression. These tissue-based experiments can be combined with an activity-based proteomics approach to identify novel protease targets and relate proteolytic activity ex vivo to the observed degradation products of extracellular matrix in vascular disease.[58] Proteolytic activity is the key for plaque destabilization and aneurysm rupture. Numerous proteases, in particular matrix metalloproteinases, have been implicated. Yet, their targets in the vasculature are incompletely understood. By identifying proteolytic cleavage products in vascular disease, proteomics could provide a valuable resource that may reveal novel biomarkers.[59] These biomarkers can then either be detected in the circulation or in urine, where proteolytic degradation products tend to accumulate.[60]

Biomarker Validation

Independent of the molecular entity of the biomarker, validation should adhere with rigor to the respective scientific statement by Hlatky et al[61] endorsed by the American Heart Association. This includes the proof of an independent and robust association in adequately sized prospective population studies, stringent replication, testing of an incremental predictive value and of clinical use involving analyses on model calibration, risk discrimination, and risk reclassification. Tests commonly applied in this context are the c-statistics/index, the net reclassification index based on clinically relevant categories of risk,[62] continuous net reclassification index,[63] prospective net reclassification index,[63] and integrated discrimination improvement.[62] On top of biomarker discovery and validation, optimal timing and frequency of risk assessment as well as the best-fitting prediction period deserve adequate attention. Ascertainment of short-term CVD risk and of an unstable plaque phenotype, using imaging, blood, and urine biomarkers, is as yet an unmet goal with high clinical priority. Finally, the best new biomarkers and respective risk score extensions should be directly compared in randomized trials for cost-effectiveness and efficacy beyond current practice.[61]

A major drawback for biomarker studies using postgenomic technologies is the lack of standardization (ie, different RNAs are used for normalization of circulating miRNAs), and there are no search algorithms for unambiguous metabolite identifcation by mass spectrometry. Also, conventional statistical tests are not suitable for highly correlated variables such as miRNAs or metabolites. As postgenomic technologies advance, it is paramount to establish minimum standards for data analysis. Much can be learned from the tools and methods applied to genetics and genomics, but there is also a need for the development and application of new methods and tools specific to postgenomic technologies.

Conclusions

Over the last decade, innovative methods opened new possibilities for harnessing the potential of postgenomic technologies to study CVD processes in clinical samples. Postgenomic technologies have the distinct advantage that read-outs at the transcriptome, proteome, and metabolome level and are closer to the phenotype of complex traits such as CVD, with contributing factors ranging from genes to environment. The aim is the integration of different levels of information to better characterize the complexity underlying cardiovascular and metabolic diseases with the goal of illuminating biology and discovering

clinical biomarkers that may help to stratify patients. For example, changes in circulating miRNAs could be related to lipidomic as well as proteomic signatures to find new regulatory mechanisms of CVD. If successful, postgenomic technologies may propel multimarker strategies in the clinics. A comprehensive noninvasive biosignature, comprising a panel of biomarkers, may serve as a potential tool in the early diagnosis and prognostication of cardiovascular risk and prioritize individuals at risk for therapeutic interventions. Nonetheless, "prediction is very difficult, especially about the future" (Nils Bohr).

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Disclosures

The authors filed a patent application related to circulating miRNAs as cardiovascular biomarkers.

References

1. Hess JW, Macdonald RP, Frederick RJ, Jones RN, Neely J, Gross D. Serum creatine phosphokinase (CPK) activity in disorders of heart and skeletal muscle. *Ann Intern Med*. 1964;61:1015–1028.
2. D'Agostino RB Sr, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, Kannel WB. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation*. 2008;117:743–753.
3. Greenland P, Alpert JS, Beller GA, et al; American College of Cardiology Foundation; American Heart Association. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2010;56:e50–103.
4. Khot UN, Khot MB, Bajzer CT, Sapp SK, Ohman EM, Brener SJ, Ellis SG, Lincoff AM, Topol EJ. Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA*. 2003;290:898–904.
5. Cooper JA, Miller GJ, Humphries SE. A comparison of the PROCAM and Framingham point-scoring systems for estimation of individual risk of coronary heart disease in the Second Northwick Park Heart Study. *Atherosclerosis*. 2005;181:93–100.
6. Gerszten RE, Wang TJ. The search for new cardiovascular biomarkers. *Nature*. 2008;451:949–952.
7. Di Angelantonio E, Chowdhury R, Sarwar N, Ray KK, Gobin R, Saleheen D, Thompson A, Gudnason V, Sattar N, Danesh J. B-type natriuretic peptides and cardiovascular risk: systematic review and meta-analysis of 40 prospective studies. *Circulation*. 2009;120:2177–2187.
8. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med*. 2011;5:673–694.
9. Kiechl S, Willeit J, Mayr M, Viehweider B, Oberhollenzer M, Kronenberg F, Wiedermann CJ, Oberthaler S, Xu Q, Witztum JL, Tsimikas S. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. *Arterioscler Thromb Vasc Biol*. 2007;27:1788–1795.
10. Hingorani AD, Sofat R, Morris RW, Whincup P, Lowe GD, Mindell J, Sattar N, Casas JP, Shah T. Is it important to measure or reduce C-reactive protein in people at risk of cardiovascular disease? *Eur Heart J*. 2012;33:2258–2264.
11. Humphries SE, Drenos F, Ken-Dror G, Talmud PJ. Coronary heart disease risk prediction in the era of genome-wide association studies: current status and what the future holds. *Circulation*. 2010;121:2235–2248.
12. Casas JP, Cooper J, Miller GJ, Hingorani AD, Humphries SE. Investigating the genetic determinants of cardiovascular disease using candidate genes and meta-analysis of association studies. *Ann Hum Genet*. 2006;70:145–169.
13. Yeboah J, McClelland RL, Polonsky TS, Burke GL, Sibley CT, O'Leary D, Carr JJ, Goff DC, Greenland P, Herrington DM. Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate-risk individuals. *JAMA*. 2012;308:788–795.
14. Pennisi E. Genomics. Encode project writes eulogy for junk DNA. *Science*. 2012;337:1159, 1161.
15. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105:10513–10518.
16. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS ONE*. 2008;3:e3694.
17. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Köppel 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.
18. 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 USA*. 2011;108:5003–5008.
19. 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.
20. Fichtlscherer S, Zeiher AM, Dimmeler S. Circulating microRNAs: biomarkers or mediators of cardiovascular diseases? *Arterioscler Thromb Vasc Biol*. 2011;31:2383–2390.
21. Engelhardt S. Small RNA biomarkers come of age. *J Am Coll Cardiol*. 2012;60:300–303.
22. Zhu H, Fan GC. Role of microRNAs in the reperfused myocardium towards post-infarct remodelling. *Cardiovasc Res*. 2012;94:284–292.
23. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. *Cardiovasc Res*. 2012;93:555–562.
24. 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.
25. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S, Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol*. 2012;60:290–299.
26. Widera C, Gupta SK, Lorenzen JM, Bang C, Bauersachs J, Bethmann K, Kempf T, Wollert KC, Thum T. Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. *J Mol Cell Cardiol*. 2011;51:872–875.
27. Eitel I, Adams V, Dieterich P, Fuernau G, de Waha S, Desch S, Schuler G, Thiele H. Relation of circulating MicroRNA-133a concentrations with myocardial damage and clinical prognosis in ST-elevation myocardial infarction. *Am Heart J*. 2012;164:706–714.
28. Ai J, Zhang R, Li Y, et al. Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction. *Biochem Biophys Res Commun*. 2010;391:73–77.
29. 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.
30. D'Alessandra Y, Devanna P, Limana F, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J*. 2010;31:2765–2773.
31. 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.
32. 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*. 2010;119:87–95.
33. 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 myocardial damage. *Circ Cardiovasc Genet*. 2011;4:446–454.
34. Oerlemans MI, Mosterd A, Dekker MS, de Vrey EA, van Mil A, Pasterkamp G, Doevendans PA, Hoes AW, Sluijter JP. Early assessment of acute coronary syndromes in the emergency department: the potential diagnostic value of circulating microRNAs. *EMBO Mol Med*. 2012;4:1176–1185.
35. Long G, Wang F, Duan Q, Chen F, Yang S, Gong W, Wang Y, Chen C, Wang DW. Human circulating microRNA-1 and microRNA-126 as potential novel indicators for acute myocardial infarction. *Int J Biol Sci*. 2012;8:811–818.
36. Devaux Y, Vausort M, Goretti E, Nazarov PV, Azuaje F, Gilson G, Corsten MF, Schroen B, Lair ML, Heymans S, Wagner DR. Use of circulating microRNAs to diagnose acute myocardial infarction. *Clin Chem*. 2012;58:559–567.
37. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Röxe T, Müller-Ardogan M, Bonauer A, Zeiher AM, Dimmeler S. Circulating microRNAs in patients with coronary artery disease. *Circ Res*. 2010;107:677–684.
38. Gao W, He HW, Wang ZM, Zhao H, Lian XQ, Wang YS, Zhu J, Yan JJ, Zhang DG, Yang ZJ, Wang LS. Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. *Lipids Health Dis*. 2012;11:55.
39. Sun X, Zhang M, Sanagawa A, Mori C, Ito S, Iwaki S, Satoh H, Fujii S. Circulating microRNA-126 in patients with coronary artery disease: correlation with LDL cholesterol. *Thromb J*. 2012;10:16.
40. 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.
41. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. *Eur J Heart Fail*. 2012;14:147–154.
42. 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.
43. Olivieri F, Antonicelli R, Lorenzi M, D'Alessandra Y, Lazzarini R, Santini G, Spazzafumo L, Lisa R, La Sala L, Galeazzi R, Recchioni R, Testa R, Pompilio G, Capogrossi MC, Procopio AD. Diagnostic potential of circulating mir-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int J Cardiol*. 2012, Epub ahead of print.
44. Zampetaki A, Mayr M. Analytical challenges and technical limitations in assessing circulating miRNAs. *Thromb Haemost*. 2012;108:592–598.
45. Zampetaki A, Mayr M. MicroRNAs in vascular and metabolic disease. *Circ Res*. 2012;110:508–522.
46. Mayr M. Metabolomics: ready for the prime time? *Circ Cardiovasc Genet*. 2008;1:58–65.
47. Watson AD. Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. *J Lipid Res*. 2006;47:2101–2111.
48. Stegemann C, Drozdov I, Shalhoub J, Humphries J, Ladroue C, Didangelos A, Baumert M, Allen M, Davies AH, Monaco C, Smith A, Xu Q, Mayr M. Comparative lipidomics profiling of human atherosclerotic plaques. *Circ Cardiovasc Genet*. 2011;4:232–242.
49. Han X, Gross RW. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev*. 2005;24:367–412.
50. Meikle PJ, Wong G, Tsorotes D, et al. Plasma lipidomic analysis of stable and unstable coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2011;31:2723–2732.
51. Mayr M, Zhang J, Greene AS, Gutterman D, Perloff J, Ping P. Proteomics-based development of biomarkers in cardiovascular disease: mechanistic, clinical, and therapeutic insights. *Mol Cell Proteomics*. 2006;5:1853–1864.
52. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*. 2002;1:845–867.
53. Jacquet S, Yin X, Sicard P, Clark J, Kanaganayagam GS, Mayr M, Marber MS. Identification of cardiac myosin-binding protein C as a candidate biomarker of myocardial infarction by proteomics analysis. *Mol Cell Proteomics*. 2009;8:2687–2699.
54. Bleijerveld OB, Wijten P, Cappadona S, et al. Deep proteome profiling of circulating granulocytes reveals bactericidal/permeability-increasing protein as a biomarker for severe atherosclerotic coronary stenosis. *J Proteome Res*. 2012;11:5235–5244.
55. Ramos-Mozo P, Madrigal-Matute J, Martinez-Pinna R, Blanco-Colio LM, Lopez JA, Camafeita E, Meilhac O, Michel JB, Aparicio C, Vega de Ceniga M, Egido J, Martín-Ventura JL. Proteomic analysis of polymorphonuclear neutrophils identifies catalase as a novel biomarker of abdominal aortic aneurysm: potential implication of oxidative stress in abdominal aortic aneurysm progression. *Arterioscler Thromb Vasc Biol*. 2011;31:3011–3019.
56. Didangelos A, Yin X, Mandal K, Baumert M, Jahangiri M, Mayr M. Proteomics characterization of extracellular space components in the human aorta. *Mol Cell Proteomics*. 2010;9:2048–2062.
57. Barallobre-Barreiro J, Didangelos A, Schoendube FA, Drozdov I, Yin X, Fernández-Caggiano M, Willeit P, Puntmann VO, Aldama-López G, Shah AM, Doménech N, Mayr M. Proteomics analysis of cardiac extracellular matrix remodeling in a porcine model of ischemia/reperfusion injury. *Circulation*. 2012;125:789–802.
58. Didangelos A, Yin X, Mandal K, Saje A, Smith A, Xu Q, Jahangiri M, Mayr M. Extracellular matrix composition and remodeling in human abdominal aortic aneurysms: a proteomics approach. *Mol Cell Proteomics*. 2011;10:M111.008128.
59. Didangelos A, Stegemann C, Mayr M. The -omics era: proteomics and lipidomics in vascular research. *Atherosclerosis*. 2012;221:12–17.
60. Zimmerli LU, Schiffer E, Zürbig P, Good DM, Kellmann M, Mouls L, Pitt AR, Coon JJ, Schmieder RE, Peter KH, Mischak H, Kolch W, Delles C, Dominiczak AF. Urinary proteomic biomarkers in coronary artery disease. *Mol Cell Proteomics*. 2008;7:290–298.
61. Hlatky MA, Greenland P, Arnett DK, et al; American Heart Association Expert Panel on Subclinical Atherosclerotic Diseases and Emerging Risk Factors and the Stroke Council. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. *Circulation*. 2009;119:2408–2416.
62. Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med*. 2008;27:157–172.
63. Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med*. 2011;30:11–21.