Trends in Molecular Medicine

CellPress REVIEWS

Review

Noncoding RNAs versus Protein Biomarkers in Cardiovascular Disease

Christian Schulte [1,2,3,@] Temo Barwari,[1] Abhishek Joshi,[1,4,@] Tanja Zeller,[2,3] and Manuel Mayr [1,*,@]

The development of more sensitive protein biomarker assays results in continuous improvements in detectability, extending the range of clinical applications to the detection of subclinical cardiovascular disease (CVD). However, these efforts have not yet led to improvements in risk assessment compared with existing risk scores. Noncoding RNAs (ncRNAs) have been assessed as biomarkers, and miRNAs have attracted most attention. More recently, other ncRNA classes have been identified, including long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs). Here, we compare emerging ncRNA biomarkers in the cardiovascular field with protein biomarkers for their potential in clinical application, focusing on myocardial injury.

Highlights

Cardiac troponins (cTns) are the gold standard biomarker for detecting acute myocardial damage. With increasing sensitivity opportunities arise for a potential application in cardiovascular risk prediction in the general population.

cMyBP-C has biomarker properties with an earlier release from injured cardiomyocytes than Tns.

Among ncRNAs, miRNAs, lncRNAs, and circRNAs have been studied in the context of cardiac injury; miRNAs are stable in the circulation and have cardiac or muscle specificity; most circulating lncRNAs may not be of cardiac origin; cardiac circRNAs show poor detectability in plasma.

ncRNA biomarker evaluation is still hampered by methodological issues such as medication as confounding factors and high analysis costs.

Necessity for Novel Biomarkers in CVD

Biomarkers have guided treatment decisions for CVD over the past 50 years (Box 1). For cardiac **troponins** (c**Tns**) (see Glossary), this is apparent in the latest and fourth universal definition of myocardial infarction (MI): for the first time myocardial injury is defined alongside myocardial infarction as a different disease entity, as a result of the ongoing improvements of assay sensitivities allowing for detection of minute changes in circulating cTn levels. Consequently, even settled elevations of cTn levels can be detected [1]. A flipside of this increase in **sensitivity** is an increased likelihood of false positives, requiring new definitions of pathological values in acute and chronic settings, or the definition of new disease states where very low levels of the detected biomarker capture a separate phase of the disease process (see Clinician's Corner). Thus, there is still a need to improve upon and complement existing biomarkers for CVD. In this respect, an approach of complementary biomarker combination seems attractive. Circulating **noncoding RNAs (ncRNAs)** are currently being assessed as alternative and complementary biomarker candidates, and initial results, especially for **miRNAs**, show potential for implementation in clinical research. Further ncRNA species, such as **long noncoding RNAs (lncRNAs)** and **circular RNAs (circRNAs)** have properties of circulating biomarkers, but their scientific exploration is still in its infancy (Figure 1).

Protein Biomarkers

Tn

Tns are a component of thin myofilaments of the contractile apparatus (together with actin and tropomyosin). The Tn complex consists of TnC, TnI, and TnT. cTnI and cTnT are cardiac-specific and among the most widely used biomarkers (Figure 1). They are the gold standard for detection of myocardial injury due to their cardiac **specificity** and high sensitivity of current assays [2]. The assay performance is a result of extensive optimization since their first identification as circulating biomarkers almost three decades ago [3]. While only a few years ago, any detectable cTn values implied a pathological process, the latest high sensitivity cTn (hs-cTn) assays return detectable values in a large proportion of healthy individuals. Before expanding the application of this assay to diagnosis of subclinical disease and risk prediction [4], it remains to be determined if such low cTn levels reflect clinically relevant myocardial injury or just

[1]King's British Heart Foundation Centre, King's College London, 125 Coldharbour Lane, London SE5 9NU, UK
[2]Clinic for Cardiology, University Heart and Vascular Center Hamburg, Hamburg, Germany
[3]German Centre of Cardiovascular Research (DZHK), Partner Site Hamburg, Luebeck, Kiel, Hamburg, Germany
[4]Bart's Heart Centre, St. Bartholomew's Hospital, West Smithfield, London, UK

*Correspondence:
manuel.mayr@kcl.ac.uk (M. Mayr).
@Twitter: @Schulte__C (C. Schulte), @CardiacJoshi (A. Joshi), @Vascular_Prot (M. Mayr).

Box 1. Historical Timeline of the Identification of Biomarkers for MI.

The first cardiac-specific protein biomarker, creatine kinase myocardial band (CK-MB) was the biomarker of choice for the detection of MI in the 1980s and 1990s [98]. Until then, the general muscle marker creatine kinase (CK) was clinically used to determine damage to any type of striated muscle. This obviously involved a high degree of uncertainty, and thus a lack of specificity, with regards to the detection of cardiac damage. In the mid-1990s, the quantification of circulating CK-MB levels allowed for determining quantifiable levels in the circulation 6–10 h upon onset of myocardial injury. Thus, measurements of CK-MB every 12 h was defined as an adequate and cost-effective method for the diagnosis of MI [98], earlier than at-the-time available cTn assays [98]. In the early 2000s, cTnI/T first complemented and later displaced CK-MB as the standard biomarker for acute MI [99], and have been the gold standard in the detection of MI ever since. Their good sensitivity and high specificity for the myocardium made them superior to CK-MB. Nevertheless, CK-MB may still be used in biomarker-based estimation of the extent of myocardial damage upon MI or after coronary intervention, as well as in patients with impaired renal function, where cTn levels can be elevated [100]. Over the past decade, significant improvements in cTn detection assays have led to the development of high-sensitivity assays, which have contributed to changes in clinical practice. The application of current hs-cTn assays allows for a sensitive detection of MI within 1 h after hospital presentation and thus improves early stratification of patients with suspected MI [101]. A timeline of biomarker discovery for MI is presented in Figure I and illustrates the novelty of RNA-based circulating biomarkers compared with protein-based biomarkers, including the novel protein biomarker candidate cMyBP-C.

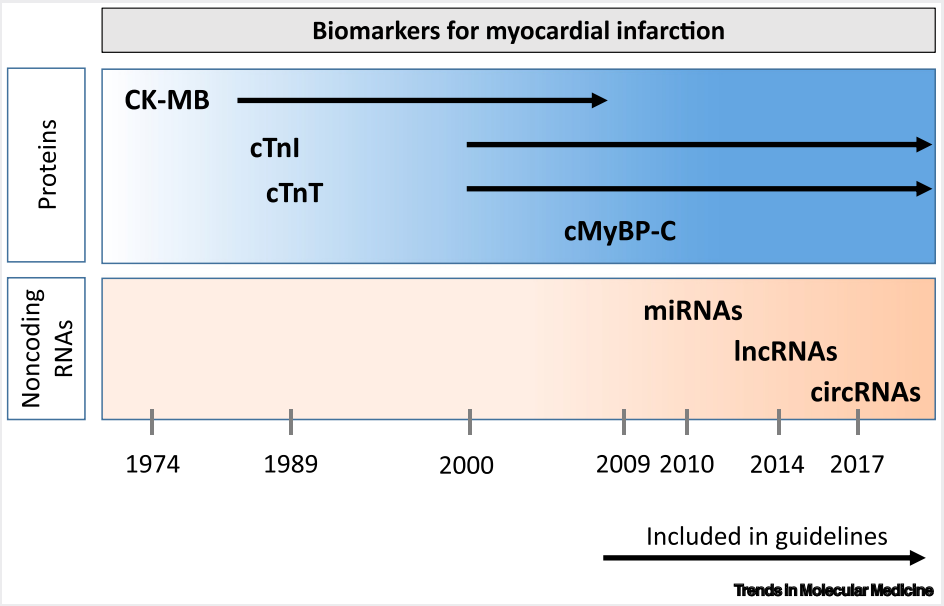

Figure I. Biomarkers for Myocardial Infarction. Abbreviations: circRNAs, circular RNAs; CK-MB, creatine kinase myocardial band; cMyBP-C, cardiac myosin-binding protein C; cTn1, cardiac troponin I; cTnT, cardiac troponin T; lncRNAs, long noncoding RNAs.

myocyte protein turnover. New cutoffs for pathological cTn values will have to be defined in acute and chronic disease settings. As an example illustrating how hs-Tn assays are transforming cTn from a diagnostic to a potential prognostic marker for CVD risk assessment [5], cTn levels were assessed in 22 000 individuals with suspected MI. Within the MI group the 1- and 2-year risk for further CVD events gradually increased with the cTn level at presentation – even at values below the 99th centile. Strikingly, the same trend was observed in 8345 matched-pair individuals from a data set comprising 70 000 individuals from the general population, exemplifying the potential use of cTn as a biomarker for primary CVD risk assessment. Yet, the use of cTn in addition to existing CVD risk scores offered little improvement upon risk assessment using conventional risk factors only [6]. Therefore, complementary biomarkers are needed.

Glossary

Area under the receiver operating curve (AUC): a statistical measure depicting the goodness of predicting an outcome, such as a diagnosis. Depicted in percent, where 100% is the perfect prediction.
Circular RNA (circRNA): ncRNA species characterized by a circular structure. Product of alternative splicing in a head-to-tail fashion known as back-splicing.
Cardiac myosin-binding protein C (cMyBP-C): a predominantly thick filament-associated functional, muscle-specific protein. The cardiac-specific isoform possesses a unique N-terminal domain, which is detectable via specific antibodies.
C-reactive protein (CRP): an acute phase reactant protein, released from the liver in inflammatory states.
Long intergenic ncRNA predicting cardiac remodeling (LIPCAR): a lncRNA.
Long noncoding RNA (lncRNA): a large group of ncRNAs defined by being >200 nucleotides in length.
miRNA: short (~22 nucleotides) ncRNA molecules.
Noncoding RNA (ncRNA): RNA that does not code for protein translation.
N-terminal pro-B-type natriuretic peptide (NT-proBNP): a protein biomarker for the diagnosis of heart failure.
Quantitative PCR (qPCR): gold standard quantification method for RNA/DNA.
Receiver operating characteristic (ROC): a performance measurement for classification of probability used to depict AUC (see above) values.
Sensitivity (of a biomarker): the fraction of individuals correctly identified as positive for a disease (using a biomarker).
Specificity (of a biomarker): the fraction of individuals correctly identified as negative for a disease (using a biomarker).
Transcoronary ablation of septal hypertrophy (TASH): interventional treatment of severe hypertrophic cardiomyopathy involving catheter-based injection of ethanol in a septal branch of the myocardium to induce myocardial cell death, and a consecutive reduction in myocardial mass to alleviate ventricular outflow obstruction.

Cardiac Myosin-Binding Protein C

cTn has revolutionized the diagnostics of acute myocardial injury over the past two decades, but with the exceptional sensitivity of the latest cTn assays there is a need for improvements in specificity. Peak cTn levels are reached 24 h after onset of MI. Cardiac biomarkers with an earlier release and faster clearance could add value in clinical decision making. **Cardiac myosin-binding protein C (cMyBP-C)** is a sarcomeric, thick filament-associated protein which is more abundant in cardiomyocytes than cTn (Figure 1) [7]. Using proteomics, it was discovered that cMyBP-C is released earlier than cTn upon myocardial injury [8,9]. Ischemia-induced proteolysis and the generation of N-terminal fragments of cMyBP-C provide a likely explanation for this earlier release. In patients undergoing **transcoronary ablation of septal hypertrophy (TASH)**, cMyBP-C rises more rapidly after onset of myocardial injury than cTn [10]. In **receiver operating characteristic (ROC)** analysis, cMyBP-C outperformed hs-cTnT and hs-cTnI after TASH [11]. Subsequent studies in patients with MI, where cTn serves as the adjudicator of the diagnosis, showed that cMyBP-C offered comparable performance with cTn [11]. As testimony to the high sensitivity of this assay, cMyBP-C was detectable in all healthy individuals serving as controls, while one-fifth had hs-cTnT levels below the lower limit of detection [11]. This suggests cMyBP-C as a candidate biomarker for early and subclinical disease states as well as for risk prediction. Anand *et al*. reported cMyBP-C to be associated with myocardial hypertrophy and fibrosis in aortic stenosis patients, and an increased risk of mortality in these patients [12]. However, no extensive data for cMyBP-C are available so far in a primary preventive setting. In this respect, the evaluation of cTn in subacute CVD entities and risk prediction has been assessed more thoroughly,

Troponin (Tn): a complex of three regulatory proteins (TnC – striated muscle; TnI and TnT – cardiac muscle-specific) essential for muscle contraction.

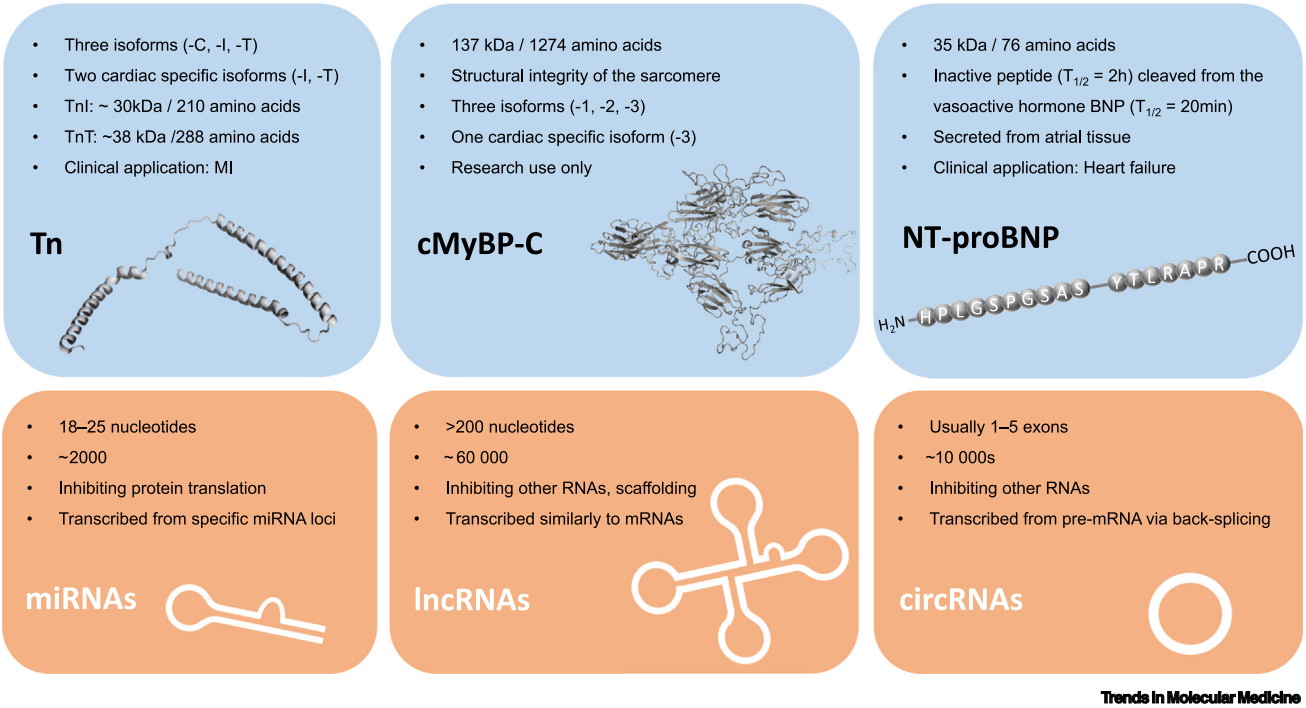

Figure 1. Main Characteristics of Cardiac Protein and ncRNA Biomarkers. cTnI and cTnT are the gold standard for myocardial injury. cMyBP-C is a larger protein. However, predominently the N terminus of cMyBP-C is detected in the circulation. NT-proBNP is an inactive metabolite derived from proBNP, which is cleaved into BNP and NT-proBNP. Among ncRNAs, miRNAs are short ncRNAs <25 nucleotides in length, with a typical hairpin shape. lncRNAs comprise a larger ncRNA family and are only vaguely defined by their length of >200 nucleotides rather than their shape. The defining criterion for circRNAs is their circularity as a product of post-transcriptional back-splicing. Abbreviations: circRNA, circular RNA; cMyBP-C, cardiac myosin-binding protein C; lncRNA, long noncoding RNA; NT-proBNP, N-terminal pro-B-type natriuretic peptide; MI, myocardial infarction; Tn, troponin.

not least because cTn assays are widely available and extensively validated. cMyBP-C measurements still need to be performed on a larger scale with defined cutoff values to address the risk of false positives that accompanies biomarkers with high sensitivity.

Growth Differentiation Factor-15 (GDF15)

cTn and cMyBP-C assays have been developed as biomarkers of acute MI, taking advantage of their cardiac specificity. GDF15 is one of the most promising candidate markers for nonacute CVD entities and risk prediction. GDF15 is a transforming growth factor β superfamily cytokine formerly named macrophage inhibitory cytokine 1. As part of a multiplex 85-biomarker discovery approach, GDF15 was found to be associated with incident heart failure (hazard ratio; HR 2.08), all-cause mortality (HR 1.96), and CVD death (HR 1.96) in 3523 individuals from the Framingham Heart Study [13]. GDF15 has been associated with inflammation, and it is highly expressed in atherosclerotic plaque macrophages [14]. Furthermore, it has been shown that GDF15 is a mediator in the expression of autophagy-relevant proteins in macrophages of atherosclerotic lesions [15]. GDF15 has been associated with cardiometabolic risk factors [16] and has been evaluated for risk stratification after non-ST elevation MI (n = 2081) [17] and ST-elevation MI (n = 741) [18], but also in stable and unstable angina pectoris [19]. This association withstood adjustment for clinical variables and other established biomarkers such as **N-terminal pro-B-type natriuretic peptide (NT-proBNP)**, **C-reactive protein (CRP)**, and cTn. In fact, there is also strong evidence for a prognostic role of GDF15 in heart failure [20], identifying it as an independent predictor of mortality. However, GDF15 lacks cardiac specificity. It might be a general marker of metabolic health rather than a biomarker with specificity for CVD [21], which might hamper its implementation in clinical practice.

NT-proBNP

NT-proBNP is an established biomarker for the diagnosis of heart failure, and has been shown to have prognostic value for CV death in this population [22]. Beyond heart failure, NT-proBNP is reported to aid CV risk prediction in the general population [23] and after MI [24]. NT-proBNP is a stable prohormone for BNP, exhibiting a longer half-life, and thus lower variability, which improves measurement reproducibility (Figure 1). NT-proBNP and BNP have defined biological roles in the disease process they diagnose; BNP is released in response to atrial stretch and results in reduced systemic vascular resistance and natriuresis, reducing circulating volume in heart failure. New therapies targeting neprilysin, the enzyme responsible for degrading BNP and thus prolonging its effects, have improved heart failure outcomes [25]. By contrast, therapy of acute decompensated heart failure did not show improved outcome when guided by NT-proBNP [26]. Nevertheless, NT-proBNP exemplifies a biomarker of high utility; it is highly sensitive, its biology is understood, and treatments which alter its levels have an impact on outcome. Therefore, NT-proBNP is a biomarker with potential for extension of its current clinical applicability from heart failure to other CVD entities and from a diagnostic to a prognostic biomarker.

High-Sensitive CRP (hs-CRP)

hs-CRP is a readout for systemic inflammation rather than CVD-specific pathomechanisms, comparable with GDF-15. Its use as a biomarker for CVD is based on the notion that atherosclerosis is a chronic inflammatory condition [27]. hs-CRP was proposed as a biomarker for guiding treatment decisions on statin therapy in prevention of CVD [28]. Thus far, guidelines only list hs-CRP as a Class IIb (weak recommendation), Level 2 (weak evidence) biomarker ‘as part of refined risk assessment in patients with an unusual or moderate CVD risk profile’, while it ‘should not be measured in asymptomatic low-risk individuals and high-risk patients to assess 10-year risk of CVD [29]. From a mechanistic point of view, Mendelian randomization studies have shown that elevated CRP levels are not causal for CVD [30]. Also, markers of vascular rather than systemic inflammation might be preferable for a biomarker for CVD [31].

ncRNA Biomarkers

Among the 20 000 protein-coding genes, surprisingly few proteins have cardiac tissue specificity: 12 proteins are considered to be cardiac-specific (Table 1, adapted from https://www.proteinatlas.org/humanproteome/tissue/heart) and some have already been implemented in clinical applications; that is, cTns and natriuretic peptides. With the completion of the Human Genome Project, an increasing number of ncRNAs have been identified. Several subcategories of ncRNAs have been described and attributed to specific biological functions in regulation of gene expression [32,33]. Moreover, ncRNAs have also been assessed for their application as circulating biomarkers in CVD [34]. Given their tissue-specific expression, a subset of ncRNAs could serve as companion biomarkers [35] to existing protein biomarkers or as signatures comprising a combination of different ncRNAs to increase specificity [36]. We discuss the potential of three different ncRNA classes for CVD: miRNAs, lncRNAs, and circRNAs. Their main characteristics are depicted in Figure 1.

miRNAs

A landmark study by Mitchell *et al.* revealed that miRNAs are not just confined to the intracellular space, but are also present in the circulation [37]. By contrast to mRNAs, circulating miRNAs are protected from degradation through several mechanisms including binding to protein complexes and lipoproteins as well as being encapsulated in microvesicles [38–40]. miRNAs function as repressors of gene expression via binding to mRNA and inhibition of protein translation [41]. However, the copy numbers of circulating miRNAs are low. Without a receptor or another amplification mechanism, it is difficult to envisage how the stoichiometry of circulating miRNAs to intracellular mRNA targets can result in significant changes in gene expression. Regardless of their function, their potential use as circulating biomarkers for CVD has been studied over the past few years [42,43].

miRNAs as CVD Biomarkers

Hundreds of miRNAs can be detected in plasma and serum samples from healthy volunteers. However, a subset of miRNAs with cardiac enrichment is only detectable in the circulation after

Table 1. Twelve Cardiac-Specific Genes with the Highest Expression in the Heart[a,b]

Gene	Protein	Clinical use as biomarker
NPPB	Natriuretic peptide B	Established: heart failure Novel/potential: CVD risk
MYL7	Myosin, light chain 7	No
NPPA	Natriuretic peptide A	No
MYBPC3	Cardiac myosin-binding protein C	Novel: diagnostic in MI Potential: CVD risk
TNNT2	Troponin T type 2 (cardiac)	Established: diagnostic in MI Novel: post-MI risk Potential: CVD risk
BMP10	Bone morphogenetic protein 10	No
TNNI3	Troponin I type 3 (cardiac)	Established: diagnostic in MI Novel: post-MI risk Potential: CVD risk
MYH6	Myosin heavy chain 6 (cardiac)	No
ANKRD1	Ankyrin repeat domain 1 (cardiac)	No
RD3L	Retinal degeneration 3-like	No
SBK2	SH3 domain binding kinase family member 2	No
MYL4	Myosin, light chain 4	No

[a]Genes are ranked by tissue specificity score (TS score), which represents the fold-change to the second highest tissue.
[b]Amended from https://www.proteinatlas.org/humanproteome/tissue/heart.

MI. miRNAs that are highly expressed in the heart include miR-1 and miR-133. Similar to myofilament proteins, miR-1 and miR-133 are also present in skeletal muscle. By contrast, miR-499 and miR-208 are less abundant in the heart but more specific for cardiac versus skeletal muscle injury [44]. A study by van Rooij *et al.* [45] described dysregulated miRNAs in failing hearts of humans and mice. A subsequent study in the context of MI showed dysregulation of miRNAs after myocardial injury [46]. Muscle- and cardiac-enriched miRNAs were low or even undetectable in plasma from healthy volunteers, whereas in patients with MI, an increase was detectable as soon as one hour after onset of ischemia [47].

To determine release of miRNAs upon myocardial injury in a well-controlled model of myocardial injury, Liebetrau *et al.* studied patients undergoing TASH [48]. As the precise onset of myocardial injury in this procedure is known, this setting allows for a well-controlled assessment of release kinetics of cardiac miRNAs upon MI. In line with previous studies, plasma levels of muscle-enriched miR-1, miR-133a, and cardiac-enriched miR-208a increased during the first 4 h after TASH. The authors also demonstrated that miRNA release kinetics correlated with cTn levels. However, cardiac biomarker release after TASH may differ from that after ischemia. Validating the findings from the TASH model, ROC analysis on the temporal release of ncRNAs and protein biomarkers in combination with a trained model of fivefold cross-validation in TASH were performed and compared with patients with acute MI as validation cohort [11]. Importantly, both cohorts returned similar **area under the receiver operating curve (AUC)** values for cardiac biomarker combinations, leading to two main conclusions: (i) the applicability of the TASH model in studying the effect of myocardial injury on biomarker release; and (ii) the potential of combinations of different biomarkers as diagnostic tools.

Considerations for Use of miRNAs as Biomarkers

With regard to the available publications on miRNAs as biomarkers for cardiac injury, there are two important considerations. First, quantification methods for miRNAs rely on real-time **quantitative PCR (qPCR)** assays. Not only are these measurements time-consuming and expensive, they are also confounded by the presence of heparin [49]. Surprisingly, even a single bolus injection of heparin is sufficient to alter the results from qPCR assays [49]. Yet, most key publications studying miRNAs as potential biomarkers for acute MI have not accounted for the presence of heparin, although it is routinely administered in cases with suspected MI (Table 2). ncRNA detection is reported to be inhibited even in conditions with high endogenous heparin levels, such as thoracic aortic aneurysm [50], but this finding awaits confirmation by others. Treatment of RNA samples with heparinase can overcome this confounding issue [51]. Second, detectability of trace amounts of cardiac miRNAs, analogous to low Tn levels, is still an unmet need. Stringent evaluation of cardiac miRNA levels in MI patients with low but elevated cTn levels measured with high-sensitivity assays returned a high proportion of undetectable results, indicating that current miRNA assays lack sensitivity compared with measurements of protein-based biomarkers for cardiac injury [11]. Yet, despite this lack of sensitivity, a combination of protein and ncRNA biomarkers showed the best performance in correctly identifying patients with acute MI, even in patients with initially low and then steeply rising corresponding hs-cTn values [11]. One potential explanation for this additive value is differences in the release kinetics from the injured myocardium, as well as differences in the clearance rate from the circulation for protein versus ncRNA biomarkers.

Finally, in addition to the release of miRNAs from cardiac tissue, acute MI is accompanied by a systemic response that may well be reflected in changes of circulating ncRNAs but is not directly the result of the myocardial injury. In order to discern the systemic response from the injury-

Table 2. Selected Publications on miRNA Biomarkers in Acute MI

Patients	ncRNAs	Protein biomarker comparison	Biomarker kinetics	Heparin accounted for[a]	ROC analysis	Year	Refs
MI vs hospital controls	miR-1	cTnI CK-MB	No	?	No	2009	[102]
ACS[b] vs CHF vs controls	miR-499	No	No	?	No	2010	[103]
MI vs controls	miR-1	CK-MB	No	?	No	2010	[104]
STEMI vs controls	miR-1 miR-133a miR-133b miR-499 miR-122 miR-375	cTnI	Yes	?	No	2010	[105]
MI vs non-MI vs controls	miR-1 miR-133a miR-208a miR-499	ROC analysis only: cTnI	No	?	Yes – single miRNA vs. cTnI	2010	[47]
MI vs myocarditis vs HF vs controls	miR-1 miR-133a miR-208b miR-499 miR-122 miR-223	Correlation only: cTnT	No	?	Yes – single miRNA vs. cTnT	2010	[106]
MI vs stable AP vs controls; coronary sinus	miR-133a miR-208a miR-499 miR-92a miR-126 miR-155 miR-223	hs-cTnT	No	Not administered	No	2011	[107]
ACS vs non-ACS	miR-1 miR-133a	cTnT	No	?	No	2011	[108]
NSTEMI vs STEMI vs controls	miR-208b miR-499	hs-cTnT	No	?	Yes – single miRNA vs. hs-cTnT	2012	[109]
MI vs controls	miR-1 miR-126	cTnI	No	?	Yes – single miRNA	2012	[110]
NSTEMI and UA vs non-ACS	miR-1 miR-208a miR-499 miR-21 miR-146a	hs-cTnT	No	?	Yes – miRNA combinations vs. hs-cTnT	2012	[111]
TASH	miR-1 miR-133a miR-208a miR-21	hs-cTnT	Yes	?	No	2013	[48]
NSTEMI vs HF vs controls	miR-1 miR-133a miR-208a miR-499 miR-21 miR-423	cTnT	No	Not administered	Yes – single miRNAs vs. hs-cTnT	2013	[112]
TASH; STEMI, NSTEMI- type 1 vs non-cardiac chest pain	Cardiac miRNAs Muscle miRNAs Plasma miRNAs -lncRNAs -circRNAs	cMyBP-C hs-cTNI hs-cTnT CK-MB CK	Yes	Heparinase treatment	Yes – single miRNAs and miRNA combinations vs. hs-cTnT, cMyBP-C and their combination	2019	[11]

[a]None of the most important publications on miRNA-application as circulating biomarkers in acute MI accounted for the confounding effect of heparin when quantifying RNA via RT-qPCR.
[b]Abbreviations: ACS, acute coronary syndrome; AP, angina pectoris; CHF, congestive heart failure; NSTEMI, non-STEMI; STEMI, ST-elevation MI; UA, unstable angina pectoris.

induced release of miRNA, spike-in of human myocardial tissue into healthy human plasma and quantification of a range of miRNAs revealed that cardiac- and muscle-enriched miRNAs -1, -133, -208, and -499 showed a linear dose–response proportional to the amount of spiked-in myocardial tissue [11]. However, no changes were detectable for other miRNAs that have previously been described as altered after MI, i.e., miR-126, miR-223 and miR-21, refuting a predominant cardiac origin. Instead, these miRNAs are highly expressed in platelets, with platelet activation by ischemia [52,53] and antiplatelet medication affecting their circulating levels in acute MI. Platelet miRNAs are unlikely to have diagnostic utility in acute MI, but may be informative with regards to CVD risk prediction [54].

The first prospective community-based cohort study examining the predictive value of circulating miRNAs with respect to MI in a primary prevention setting was performed by Zampetaki *et al.* [55]. The authors found a signature of three miRNAs, platelet-enriched miR-223 and miR-197, as well as endothelial-cell- and platelet-enriched miR-126. Importantly, none of these miRNAs were predictive on their own, but instead only their combination returned significant associations with future CVD events. The prognostic value of these three miRNAs as circulating biomarkers was further explored in a cohort of 873 patients with diagnosed coronary artery disease (CAD) in a secondary prevention setting. Unlike in primary prevention, a combination of miRNAs was not required: elevated levels of miR-126 were predictive for future CV death [54,56]. miR-197 and miR-223 were also positively associated with an adverse outcome [56]. It is plausible that antiplatelet treatment in secondary prevention influences circulating miR-126, miR-197, and miR-223 levels. Higher levels of these miRNAs may reflect less-efficient platelet inhibition on antiplatelet therapy. Bye *et al.* evaluated several miRNAs as potential predictors of future MI, proposing a panel of five miRNAs that could enhance the predictive strength of conventional algorithms [57]. The authors were not able to validate the above-mentioned, previously identified [55] platelet and endothelial cell miRNAs as predictors of CVD outcome. As a potential reason, the authors discuss different normalization methods applied in the studies. Altogether, these studies indicate that: (i) miRNAs could be informative on *in vivo* platelet activation or treatment response to antiplatelet therapy; and (ii) emphasize the importance of harmonized miRNA quantification methods in an attempt to facilitate comparability across studies.

lncRNAs

lncRNAs are a large group of ncRNAs defined by being >200 nucleotides in length [58] (Figure 2). Thus far, there is no agreement on subclassification of lncRNAs. Unlike miRNAs, lncRNAs are mainly located within the nucleus or mitochondria [59,60]. Their biosynthesis shares similarities with mRNAs with regard to transcription, polyadenylation, capping, and splicing [61]. For the majority of identified lncRNAs, their function remains unclear. Nuclear lncRNAs are involved in regulation of neighboring loci through transcriptional regulation or by inhibiting expression of a gene through sequestration of transcription factors [62]. Conversely, other lncRNAs enhance gene transcription. Overall, they could provide a scaffold for organizing chromatin remodeling [63].

Several lncRNAs are also detected in the circulation. This either indicates the presence of protective mechanisms against RNase-mediated degradation similar to those of miRNAs [58] or an abundant source with constant release. The plasma level of **long intergenic ncRNA predicting cardiac remodeling (LIPCAR)** was found to predict adverse cardiac remodeling and death after MI [60]. However, LIPCAR was neither dysregulated in the acute setting of MI nor after TASH, refuting a primary cardiac origin [11]. When considering the fact that LIPCAR is derived from the mitochondrial genome, it seems likely that other cells and tissues contribute to circulating LIPCAR levels, such as platelets, leukocytes and erythrocytes [64]. To further exemplify this issue, recently, two circulating lncRNAs (ZFAS1 and lCDR1AS) were discovered as independent

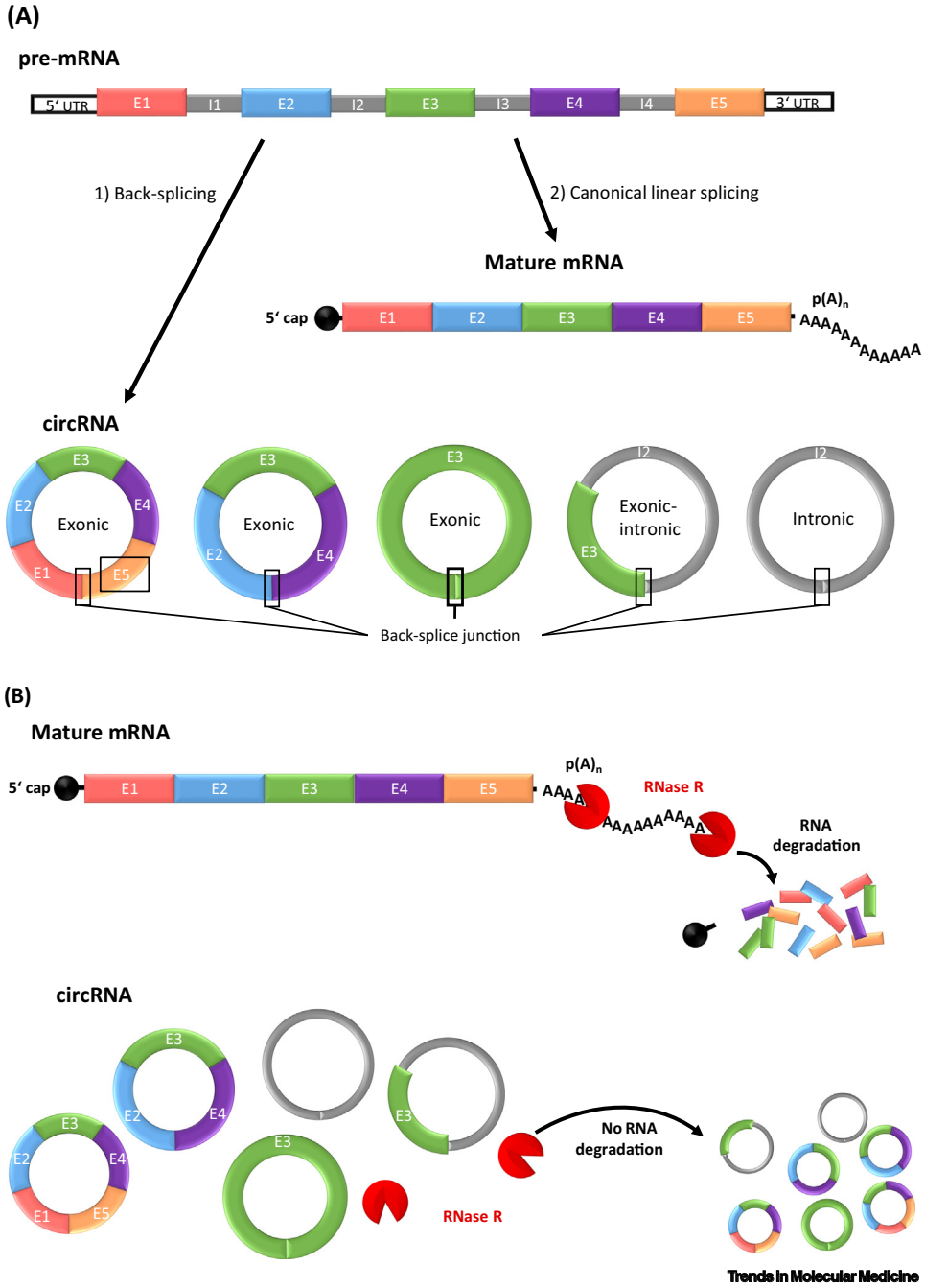

Figure 2. circRNAs Are Generated via Back-Splicing and Are Resistant to RNase R Degradation. (A) Both, mRNA and circRNA are spliced from pre-mRNA. circRNA generation requires the specific process of back-splicing in order to achieve the generation of a circular shape with the 3′ and 5′ ends joining in a back-splice junction, where the two free ends are joined to form a complete circle. circRNAs can be made up either exclusively of exons or of exons and introns or of an intron only. (B) mRNA contains a poly-A tail that is targeted by RNase R to cause degradation. circRNAs do not contain poly-A tails and are therefore protected from this particular way of degradation, which is used to prove the circularity of a targeted RNA by degradation of all linear transcripts. Abbreviations: circRNA, circular RNA; lncRNA, long noncoding RNA.

predictors for MI [65]. However, since the lncRNAs were derived from whole blood, their origin or mechanistic link to MI remains unclear. Furthermore, lower levels of lncRNA HOTAIR were found in plasma of MI patients [66] and were described to be cardioprotective through interacting with miR-1. Nevertheless, lower levels of a lncRNA after MI again question its cardiac origin. It is a common misconception that ncRNA changes in disease are related to the diseased tissue. In many cases, these reflect secondary changes, that is, due to medication, inflammatory responses, or differences in sampling (peripheral venous blood versus arterial blood collected during coronary angiography). Besides the above-mentioned lncRNAs, additional transcripts have been suggested as potential circulating biomarkers in CVD [58,67,68]. Many of the reported findings of lncRNAs as biomarkers for CVD are confined to small cohorts and require independent validation.

circRNAs

circRNAs are endogenous to mammalian cells and expressed in a tissue- and development-specific context [69,70]. They can either emerge from exons or introns of primary gene transcripts (pre-mRNA) [70,71] and are products of alternative splicing in a head-to-tail fashion known as back-splicing [69] (Figure 2A). circRNAs are resistant to degradation by the exonuclease RNase R – a type of RNase that cleaves linear RNA (Figure 2B). RNase R treatment is therefore used to enrich circRNAs over their linear counterparts [72,73]. In combination with the use of divergent primers in PCR amplification, this approach ensures specificity for the detection of circular transcripts.

Functionally, circRNAs may act as miRNA sponges – thereby decreasing the inhibitory effect of miRNAs on protein synthesis [74]. There are few circRNAs with many miRNA binding sites. More recently, circRNAs were reported to be translated into proteins [75]. At the same time, their expression is regulated by proteins such as RNA-binding proteins. circRNAs appear to influence gene expression by competing with splicing of their linear counterparts [73,76,77]. circRNA expression occurs in a tissue- and cell-specific manner, while also showing some degree of conservation across species [76,78].

circRNAs as Biomarkers in MI

Sequencing data revealed the presence of >15 000 circRNAs in the human heart; some in high abundance [79]. While intron-derived circRNAs are primarily found in the nucleus [74], the majority of circRNAs, in particular those with exonic origin (Figure 2A), are located in the cytoplasm [80]. This may increase the likelihood of their early release into the circulation upon myocardial injury. Some studies have also described a mechanistic role for cardiac circRNAs in MI-induced apoptosis [81,82]. When evaluating circRNAs as potential biomarkers for acute MI [11], several candidate circRNAs that were detectable in cardiac tissue were undetectable in cell-free plasma or serum even after extensive myocardial injury such as TASH. Other reports implicate circulating circRNAs as biomarkers for CVD in less-acute settings [83,84]. The authors did not use plasma or serum samples but instead used RNA isolated from whole blood, increasing the likelihood of the detected transcripts being blood cell derived. Similar to LIPCAR, the findings may reflect changes in inflammatory or other hematopoietic cells either as a potential consequence of a systemic response after MI [83] or effects of medication. Other studies evaluating circRNAs as biomarkers were either small in size [85], or performed normalization using a transcript of a commonly used intracellular reference gene [86]. The latter results could reflect plasma levels of residual blood cells such as platelets or erythrocytes. Overall, circRNAs are difficult to detect in cell-free body fluids, which currently hampers their evaluation as biomarkers. However, circRNA detection assays are in their infancy and improvements may lead to better strategies in their evaluation as circulating biomarkers.

Clinician's Corner

hs-cTn assays have facilitated the diagnosis of acute MI. However, higher sensitivity has resulted in reduced specificity.

cMyBP-C has recently been reported as a cardiac-specific and sensitive biomarker for the detection of MI.

ncRNAs, comprising miRNAs, lncRNAs and circRNAs, are alternative biomarker candidates.

A clinical application of ncRNAs as circulating biomarkers is currently not feasible.

The complimentary use of biomarkers with different release characteristics may improve the diagnosis and/or risk assessment of CVD.

Challenges in Implementing ncRNA Assessment in Biomarker Research

Current protein biomarker assays (i.e., current hs-cTn) have been improved over the past 30 years. Despite advances in ncRNA research, ncRNA detection is still inferior compared with detection of cardiac proteins [11]. Furthermore, the lack of reproducibility of published ncRNA data is a concern. Tissue-specific miRNAs, such as liver-derived miR-122, show consistent results across studies in obesity, diabetes, metabolic syndrome, and liver injury [87,88]. However, this is not the case for other miRNAs, probably because they are blood cell-derived and underlie a higher influence by preanalytical variation. Some of the major sources of variation are sample preparation and storage conditions, control of RNA degradation [89], presence of residual cells in body fluids [90], and the effect of hemolysis on ncRNA levels [91]. Furthermore, different RNA isolation methods [92], normalization methods, and the selection of the platform for ncRNA quantification [93] can impact on the quantification of circulating ncRNAs. In particular, user-dependent parameters such as detection thresholds and calibration methods contribute to inconsistencies in ncRNA quantification. Other confounders in ncRNA biomarker research are medication related, such as heparin [94] and antiplatelet drugs [95]. Attempts have been made to harmonize the reporting of ncRNA methods in the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines [96] but the guidelines are not yet widely implemented [97]. Additionally, ncRNA quantification, including RNA isolation, is still not sufficiently automated and too time consuming for an application in acute clinical settings.

Concluding Remarks

Currently, biomarkers are the domain of proteins. One of the most widely used protein biomarkers is cTn – the gold standard for the detection of early myocardial injury and MI. Apart from its use in the acute setting of MI, cTn is now also evaluated as a biomarker for nonacute CVD and risk stratification. cTn is already being used in clinical routine worldwide. Nevertheless, novel CV biomarker candidates are being explored. cMyBP-C shows properties as an early rule-in/rule-out cardiac biomarker in the acute setting of MI. The high correlation of cTn and cMyBP-C may indicate some redundancy in that both markers provide similar information. However, hs cTn assays also give rise to false-positive results. Further studies and validation in existing clinical trials are warranted to determine whether this can be mitigated by additional cMyBP-C measurements.

While both cTn and cMyBP-C are cardiac-specific, markers of metabolic and inflammatory processes could contribute to diagnostic and prognostic performance in terms of early detection and higher predictive power; that is, GDF-15, NT-proBNP, or CRP have been implicated as prognostic biomarkers for CVD. However, their added value compared with existing CVD risk scores is still limited (see Outstanding Questions). In this respect, circulating ncRNAs may be useful in complementing protein biomarkers, especially with respect to specificity, given their distinct tissue and organ enrichment combined with the large number of ncRNA transcripts expressed in different cell and tissue types. At present, miRNAs are the most promising ncRNA species. Over the past decade numerous studies have investigated miRNAs as diagnostic and prognostic circulating biomarker candidates for CVD. Nevertheless, validation of results is scarce, reflecting a need for harmonization of ncRNA quantification and reporting methods. Furthermore, current hs-cTn assays still outperform miRNA measurements in terms of sensitivity. In parallel, confounding factors such as qPCR inhibition by heparin need to be addressed by applying appropriate methods such as heparinase treatment.

ncRNAs such as lncRNAs as well as circRNAs have only recently attracted attention as biomarker candidates but there is less evidence compared with miRNAs. While many more lncRNA transcripts have been discovered compared with miRNAs, their evaluation as circulating biomarkers has only just begun. In this respect, initial results are promising in terms of detectability of some

Outstanding Questions

Can novel biomarkers complement the established protein biomarkers (cTn and NT-proBNP) for CVD?

Besides cardiac-specific molecules, is there value in systemic markers, that is, of inflammation or metabolism such as hs-CRP and GDF15?

Can circulating ncRNAs in body fluids become a novel source of biomarker candidates?

Which biomarkers and molecular species (proteins and/or ncRNAs) will provide additional value compared with existing risk scores?

lncRNAs in the circulation. For circRNAs, poor detectability in body fluids currently is the limiting factor for advances in the biomarker field. However, at a cellular level circRNAs are promising biomarker candidates in the context of a liquid biopsy, evaluating the association of platelet- or leukocyte-derived transcripts to CVD.

Prior to large-scale clinical evaluation and application of extracellular ncRNAs as biomarkers, several major obstacles need to be overcome, including the need for automation and scaling of measurements and improving means of detection (see Clinician's Corner).

Acknowledgements

C.S. is the recipient of a research fellowship by the Deutsche Forschungsgemeinschaft (DFG) (SCHU 2983/1-1 and SCHU 2983/2-1). T.B. was funded by a BHF Interdisciplinary PhD studentship. A.J. is a BHF Clinical Research Training Fellow (FS/16/32/32184). T.Z. is funded by the German Centre for Cardiovascular Research (DZHK) (81Z0710102). M.M. is a British Heart Foundation (BHF) Chair Holder (CH/16/3/32406) with BHF programme grant support (RG/16/14/32397). M.M was awarded a BHF Special Project grant to participate in the ERA-CVD Transnational Grant 'MacroERA: Non-coding RNAs in cardiac macrophages and their role in heart failure' and is part of the Marie Skłodowska-Curie Innovative Training Network TRAIN-HEART (http://train-heart.eu) as well as a network on 'MicroRNA-based Therapeutic Strategies in Vascular Disease' funded by the Foundation Leducq. This work was supported by the National Institute of Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS (National Health Service) Foundation Trust and King's College London in partnership with King's College Hospital and by VASCage - Research Centre on Vascular Ageing and Stroke. As a COMET centre VASCage is funded within the COMET program - Competence Centers for Excellent Technologies by the Austrian Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology, the Austrian Ministry for Digital and Economic Affairs and the federal states Tyrol, Salzburg and Vienna. We thank Ms Ella Reed for her assistance in making the graphical illustrations.

Disclosure Statement

M.M. is named inventor on a licensed patent held by King's College London for the detection of cMyBP-C as a biomarker of myocardial injury (EP2430453B1, US8546089). M.M filed and licensed patent applications on miRNAs as biomarkers (EP15193448.6, EP2776580 B1, DE112013006129T5, GB2524692A, EP2576826 B, JP2013-513740).

References

1. Thygesen, K. *et al.* (2019) Fourth universal definition of myocardial infarction (2018). *Eur. Heart J.* 40, 237–269
2. Roffi, M. *et al.* (2016) 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. *Eur. Heart J.* 37, 267–315
3. Katus, H.A. *et al.* (1989) Enzyme linked immuno assay of cardiac troponin T for the detection of acute myocardial infarction in patients. *J. Mol. Cell. Cardiol.* 21, 1349–1353
4. Ford, I. *et al.* (2016) High-sensitivity cardiac troponin, statin therapy, and risk of coronary heart disease. *J. Am. Coll. Cardiol.* 68, 2719–2728
5. Neumann, J.T. *et al.* (2019) Application of high-sensitivity troponin in suspected myocardial infarction. *N. Engl. J. Med.* 380, 2529–2540
6. Blankenberg, S. *et al.* (2016) Troponin I and cardiovascular risk prediction in the general population: the BiomarCaRE consortium. *Eur. Heart J.* 37, 2428–2437
7. Aye, T.T. *et al.* (2010) Proteome-wide protein concentrations in the human heart. *Mol. BioSyst.* 6, 1917
8. Jacquet, S. *et al.* (2009) Identification of cardiac myosin-binding protein C as a candidate biomarker of myocardial infarction by proteomics analysis. *Mol. Cell. Proteomics* 8, 2687–2699
9. Kaier, T.E. *et al.* (2017) Direct comparison of cardiac myosin-binding protein C with cardiac troponins for the early diagnosis of acute myocardial infarction. *Circulation* 136, 1495–1508
10. Baker, J.O. *et al.* (2015) Cardiac myosin-binding protein C: a potential early biomarker of myocardial injury. *Basic Res. Cardiol.* 110, 23
11. Schulte, C. *et al.* (2019) Comparative analysis of circulating noncoding RNAs versus protein biomarkers in the detection of myocardial injury. *Circ. Res.* 125, 328–340
12. Anand, A. *et al.* (2018) Cardiac myosin-binding protein C is a novel marker of myocardial injury and fibrosis in aortic stenosis. *Heart* 104, 1101–1108
13. Ho, J.E. *et al.* (2018) Protein biomarkers of cardiovascular disease and mortality in the community. *J. Am. Heart Assoc.* 7, e008108
14. Bootcov, M.R. *et al.* (1997) MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11514–11519
15. Ackermann, K. *et al.* (2019) Growth differentiation factor-15 regulates oxLDL-induced lipid homeostasis and autophagy in human macrophages. *Atherosclerosis* 281, 128–136
16. Ho, J.E. *et al.* (2012) Clinical and genetic correlates of growth differentiation factor 15 in the community. *Clin. Chem.* 58, 1582–1591
17. Wollert, K.C. *et al.* (2007) Prognostic value of growth-differentiation factor-15 in patients with non-ST-elevation acute coronary syndrome. *Circulation* 115, 962–971
18. Kempf, T. *et al.* (2007) Growth-differentiation factor-15 improves risk stratification in ST-segment elevation myocardial infarction. *Eur. Heart J.* 28, 2858–2865
19. Kempf, T. *et al.* (2009) Growth-differentiation factor-15 for risk stratification in patients with stable and unstable coronary heart disease: results from the AtheroGene study. *Circ. Cardiovasc. Genet.* 2, 286–292
20. Anand, I.S. *et al.* (2010) Serial measurement of growth-differentiation factor-15 in heart failure: relation to disease severity and prognosis in the Valsartan Heart Failure Trial. *Circulation* 122, 1387–1395
21. Cardoso, A.L. *et al.* (2018) Towards frailty biomarkers: candidates from genes and pathways regulated in aging and age-related diseases. *Ageing Res. Rev.* 47, 214–277

22. Myhre, P.L. *et al.* (2019) B-type natriuretic peptide during treatment with sacubitril/valsartan. *J. Am. Coll. Cardiol.* 73, 1264–1272
23. Frary, C.E. *et al.* (2019) Circulating biomarkers for long-term cardiovascular risk stratification in apparently healthy individuals from the MONICA 10 cohort. *Eur. J. Prev. Cardiol.* Published online November 12, 2019. https://doi.org/10.1177/2047487319885457
24. Oemrawsingh, R.M. *et al.* (2019) High-frequency biomarker measurements of troponin, NT-proBNP, and C-reactive protein for prediction of new coronary events after acute coronary syndrome. *Circulation* 139, 134–136
25. McMurray, J.J.V. *et al.* (2014) Angiotensin–neprilysin inhibition versus enalapril in heart failure. *N. Engl. J. Med.* 371, 993–1004
26. Stienen, S. *et al.* (2018) NT-proBNP (N-terminal pro-B-type natriuretic peptide)-guided therapy in acute decompensated heart failure PRIMA II randomized controlled trial (Can NT-ProBNP-guided therapy during hospital admission for acute decompensated heart failure reduce mortality and readmissions?). *Circulation* 137, 1671–1683
27. Ridker, P.M. *et al.* (2000) C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N. Engl. J. Med.* 342, 836–843
28. Ridker, P.M. *et al.* (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N. Engl. J. Med.* 359, 2195–2207
29. Perk, J. *et al.* (2012) European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). *Eur. Heart J.* 33, 1635–1701
30. Zhuang, Q. *et al.* (2019) Association of high sensitive C-reactive protein with coronary heart disease: a Mendelian randomization study. *BMC Med. Genet.* 20, 170
31. Langley, S.R. *et al.* (2017) Extracellular matrix proteomics identifies molecular signature of symptomatic carotid plaques. *J. Clin. Invest.* 127, 1546–1560
32. Ransohoff, J.D. *et al.* (2018) The functions and unique features of long intergenic non-coding RNA. *Nat. Rev. Mol. Cell Biol.* 19, 143–157
33. Williamson, L. *et al.* (2017) UV irradiation induces a non-coding RNA that functionally opposes the protein encoded by the same gene. *Cell* 168, 843–855.e13
34. E, S. *et al.* (2018) The circulating non-coding RNA landscape for biomarker research: lessons and prospects from cardiovascular diseases. *Acta Pharmacol. Sin.* 39, 1085–1099
35. Condorelli, G. and van Rooij, E. (2019) MicroRNAs as companion biomarkers for the diagnosis and prognosis of acute coronary syndromes. *Circ. Res.* 125, 341–342
36. Zeller, T. *et al.* (2014) Assessment of microRNAs in patients with unstable angina pectoris. *Eur. Heart J.* 35, 2106–2114
37. Mitchell, P.S. *et al.* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10513–10518
38. Arroyo, J.D. *et al.* (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U. S. A.* 108, 5003–5008
39. Vickers, K.C. *et al.* (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* 13, 423–433
40. Turchinovich, A. *et al.* (2011) Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 39, 7223–7233
41. Baek, D. *et al.* (2008) The impact of microRNAs on protein output. *Nature* 455, 64–71
42. Reid, G. *et al.* (2011) Circulating microRNAs: association with disease and potential use as biomarkers. *Crit. Rev. Oncol. Hematol.* 80, 193–208
43. Goretti, E. *et al.* (2014) miRNAs as biomarkers of myocardial infarction: a step forward towards personalized medicine? *Trends Mol. Med.* 20, 716–725
44. Kaudewitz, D. *et al.* (2015) MicroRNA biomarkers for coronary artery disease? *Curr. Atheroscler. Rep.* 17, 70
45. van Rooij, E. *et al.* (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. U. S. A.* 103, 18255–18260
46. van Rooij, E. *et al.* (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13027–13032
47. Wang, G.-K. *et al.* (2010) Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur. Heart J.* 31, 659–666
48. Liebetrau, C. *et al.* (2013) Release kinetics of circulating muscle-enriched microRNAs in patients undergoing transcoronary ablation of septal hypertrophy. *J. Am. Coll. Cardiol.* 62, 992–998
49. Kaudewitz, D. *et al.* (2013) Impact of intravenous heparin on quantification of circulating microRNAs in patients with coronary artery disease. *Thromb. Haemost.* 110, 609–615
50. Boileau, A. *et al.* (2018) Endogenous heparin interferes with quantification of microRNAs by RT-qPCR. *Clin. Chem.* 64, 863–865
51. Coelho-Lima, J. *et al.* (2018) Overcoming heparin-associated RT-qPCR inhibition and normalization issues for microRNA quantification in patients with acute myocardial infarction. *Thromb. Haemost.* 118, 1257–1269
52. Bakogiannis, C. *et al.* (2017) Platelet-derived chemokines in inflammation and atherosclerosis. *Cytokine* 122, 154157
53. Ziegler, M. *et al.* (2019) Platelets in cardiac ischaemia/reperfusion injury – a promising therapeutic target. *Cardiovasc. Res.* 115, 1178–1188
54. Jansen, F. *et al.* (2014) MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease. *J. Am. Heart Assoc.* 3, e001249
55. Zampetaki, A. *et al.* (2012) Prospective study on circulating microRNAs and risk of myocardial infarction. *J. Am. Coll. Cardiol.* 60, 290–299
56. Schulte, C. *et al.* (2015) miRNA-197 and miRNA-223 predict cardiovascular death in a cohort of patients with symptomatic coronary artery disease. *PLoS One* 10, e0145930
57. Bye, A. *et al.* (2016) Circulating microRNAs predict future fatal myocardial infarction in healthy individuals – the HUNT study. *J. Mol. Cell. Cardiol.* 97, 162–168
58. Viereck, J. and Thum, T. (2017) Circulating noncoding RNAs as biomarkers of cardiovascular disease and injury. *Circ. Res.* 120, 381–399
59. Derrien, T. *et al.* (2012) The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res.* 22, 1775–1789
60. Kumarswamy, R. *et al.* (2014) Circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure. *Circ. Res.* 114, 1569–1575
61. Wilusz, J.E. *et al.* (2009) Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev.* 23, 1494–1504
62. Gangwar, R.S. *et al.* (2018) Noncoding RNAs in cardiovascular disease: pathological relevance and emerging role as biomarkers and therapeutics. *Am. J. Hypertens.* 31, 150–165
63. Zampetaki, A. and Mayr, M. (2017) Long noncoding RNAs and angiogenesis: regulatory information for chromatin remodeling. *Circulation* 136, 80–82
64. Schulte, C. *et al.* (2019) Response by Schulte et al to letter regarding article, "comparative analysis of circulating noncoding RNAs versus protein biomarkers in the detection of myocardial injury." *Circ. Res.* 125, e22–e23
65. Zhang, Y. *et al.* (2016) Reciprocal changes of circulating long non-coding RNAs ZFAS1 and CDR1AS predict acute myocardial infarction. *Sci. Rep.* 6, 22384
66. Gao, L. *et al.* (2017) Circulating long noncoding RNA HOTAIR is an essential mediator of acute myocardial infarction. *Cell. Physiol. Biochem.* 44, 1497–1508
67. Hobuß, L. *et al.* (2019) Long non-coding RNAs: at the heart of cardiac dysfunction? *Front. Physiol.* 10, 30
68. Busch, A. *et al.* (2016) Prospective and therapeutic screening value of non-coding RNA as biomarkers in cardiovascular disease. *Ann. Transl. Med.* 4, 1–12
69. Jeck, W.R. and Sharpless, N.E. (2014) Detecting and characterizing circular RNAs. *Nat. Biotechnol.* 32, 453–461
70. Memczak, S. *et al.* (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338

71. Zhang, Y. *et al.* (2013) Circular intronic long noncoding RNAs. *Mol. Cell* 51, 792–806
72. Suzuki, H. *et al.* (2006) Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing. *Nucleic Acids Res.* 34, e63
73. Khan, M.A.F. *et al.* (2016) RBM20 regulates circular RNA production from the titin gene. *Circ. Res.* 119, 996–1003
74. Hansen, T.B. *et al.* (2013) Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388
75. Legnini, I. *et al.* (2017) Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. *Mol. Cell* 66, 22–37.e9
76. Rybak-Wolf, A. *et al.* (2015) Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol. Cell* 58, 870–885
77. Ashwal-Fluss, R. *et al.* (2014) circRNA biogenesis competes with pre-mRNA splicing. *Mol. Cell* 56, 55–66
78. Barrett, S.P. and Salzman, J. (2016) Circular RNAs: analysis, expression and potential functions. *Development* 143, 1838–1847
79. Tan, W.L.W. *et al.* (2017) A landscape of circular RNA expression in the human heart. *Cardiovasc. Res.* 113, cvw250
80. Jeck, W.R. *et al.* (2013) Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 19, 141–157
81. Wang, K. *et al.* (2017) Circular RNA mediates cardiomyocyte death via miRNA-dependent upregulation of MTP18 expression. *Cell Death Differ.* 24, 1111–1120
82. Geng, H.-H. *et al.* (2016) The circular RNA Cdr1as promotes myocardial infarction by mediating the regulation of miR-7a on its target genes expression. *PLoS One* 11, e0151753
83. Vausort, M. *et al.* (2016) Myocardial infarction-associated circular RNA predicting left ventricular dysfunction. *J. Am. Coll. Cardiol.* 68, 1247–1248
84. Salgado-Somoza, A. *et al.* (2017) The circular RNA MICRA for risk stratification after myocardial infarction. *Int. J. Cardiol. Heart Vasc.* 17, 33–36
85. Pan, R.-Y. *et al.* (2017) Circular RNAs promote TRPM3 expression by inhibiting hsa-miR-130a-3p in coronary artery disease patients. *Oncotarget* 8, 60280–60290
86. Wu, N. *et al.* (2017) Profiling and bioinformatics analyses reveal differential circular RNA expression in hypertensive patients. *Clin. Exp. Hypertens.* 39, 454–459
87. Willeit, P. *et al.* (2017) Circulating microRNA-122 is associated with the risk of new-onset metabolic syndrome and type 2 diabetes. *Diabetes* 66, 347–357
88. Shah, R. *et al.* (2017) Extracellular RNAs are associated with insulin resistance and metabolic phenotypes. *Diabetes Care* 40, 546–553
89. Ludwig, N. *et al.* (2018) Small ncRNA-Seq results of human tissues: variations depending on sample integrity. *Clin. Chem.* 64, 1074–1084
90. Tung, S.L. *et al.* (2018) Regulatory T cell-derived extracellular vesicles modify dendritic cell function. *Sci. Rep.* 8, 6065
91. Kirschner, M.B. *et al.* (2013) The impact of hemolysis on cell-free microRNA biomarkers. *Front. Genet.* 4, 94
92. Witwer, K.W. (2015) Circulating microRNA biomarker studies: pitfalls and potential solutions. *Clin. Chem.* 61, 56–63
93. Alikian, M. *et al.* (2017) RT-qPCR and RT-digital PCR: a comparison of different platforms for the evaluation of residual disease in chronic myeloid leukemia. *Clin. Chem.* 63, 525–531
94. Satsangi, J. *et al.* (1994) Effect of heparin on polymerase chain reaction. *Lancet* 343, 1509–1510
95. Sunderland, N. *et al.* (2017) MicroRNA biomarkers and platelet reactivity. *Circ. Res.* 120, 418–435
96. Bustin, S.A. *et al.* (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55, 611–622
97. Bustin, S.A. *et al.* (2013) The need for transparency and good practices in the qPCR literature. *Nat. Methods* 10, 1063–1067
98. Ellis, A.K. (1991) Serum protein measurements and the diagnosis of acute myocardial infarction. *Circulation* 83, 1107–1109
99. Jaffe, A.S. *et al.* (2006) Biomarkers in acute cardiac disease. the present and the future. *J. Am. Coll. Cardiol.* 48, 1–11
100. Adams, J.E. *et al.* (1993) Biochemical markers of myocardial injury: is MB creatine kinase the choice for the 1990s? *Circulation* 88, 750–763
101. Chew, D.P. *et al.* (2019) A randomized trial of a 1-hour troponin T protocol in suspected acute coronary syndromes: The Rapid Assessment of Possible Acute Coronary Syndrome in the Emergency Department With High-Sensitivity Troponin T Study (RAPID-TnT). *Circulation* 140, 1543–1556
102. Ai, J. *et al.* (2010) Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction. *Biochem. Biophys. Res. Commun.* 391, 73–77
103. Adachi, T. *et al.* (2010) Plasma microRNA-499 as a biomarker of acute myocardial infarction. *Clin. Chem.* 56, 1183–1185
104. Cheng, Y. *et al.* (2010) A translational study of circulating cell-free microRNA-1 in acute myocardial infarction. *Clin. Sci. (Lond.)* 119, 87–95
105. D'Alessandra, Y. *et al.* (2010) Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur. Heart J.* 31, 2765–2773
106. Corsten, M.F. *et al.* (2010) Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ. Cardiovasc. Genet.* 3, 499–506
107. De Rosa, S. *et al.* (2011) Transcoronary concentration gradients of circulating microRNAs. *Circulation* 124, 1936–1944
108. Kuwabara, Y. *et al.* (2011) Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ. Cardiovasc. Genet.* 4, 446–454
109. Devaux, Y. *et al.* (2012) Use of circulating microRNAs to diagnose acute myocardial infarction. *Clin. Chem.* 58, 559–567
110. Long, G. *et al.* (2012) Human circulating microRNA-1 and microRNA-126 as potential novel indicators for acute myocardial infarction. *Int. J. Biol. Sci.* 8, 811–818
111. Oerlemans, M.I.F.J. *et al.* (2012) Early assessment of acute coronary syndromes in the emergency department: the potential diagnostic value of circulating microRNAs. *EMBO Mol. Med.* 4, 1176–1185
112. Olivieri, F. *et al.* (2013) Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int. J. Cardiol.* 167, 531–536