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REVIEW

Circular RNAs in heart failure

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Cardiovascular disease, and particularly heart failure, is still a serious health care issue for which novel treatments and biomarkers are needed. The RNA family comprises different subgroups, among which the small-sized microRNAs and the larger long non-coding RNAs have shown some potential to aid in moving personalized health care of heart failure patients a step forward. Here, members of the Cardiolinc network review the recent findings suggesting that the less well-known circular RNAs may constitute a novel reservoir of therapeutic targets and biomarkers of heart failure. The knowledge of the mode of biogenesis of circular RNAs will first be reported, followed by a description of different features that make these RNA molecules of interest for the heart failure community. The functions of circular RNAs in the heart will be described, with some emphasis given to their regulation in the failing heart. Circulating in the bloodstream, circular RNAs have appeared as potential biomarkers and recent findings associated with the use of circular RNAs as heart failure biomarkers will be discussed. Finally, some directions for future research will be provided.

Keywords Heart failure • Transcriptomics • Non-coding RNAs • Circular RNAs • Biomarkers • Therapeutic targets

Introduction

Heart failure (HF) represents one of the major challenges facing health care systems in industrialized societies, and an increasing burden in developing countries. The management of HF is guided by the results of large, well-conducted clinical trials, the findings of which form the basis of European[1] and other guideline documents.[2] Recently developed pharmacological therapies show benefit compared with older treatments.[3] However, with contemporary management, the outlook for patients with HF remains poor. In the multinational European Society of Cardiology and Heart Failure Association HF Long-Term Registry, in-hospital and 1-year all-cause mortality following hospitalization for acute HF is 5.6% and 25.7%, respectively.[4] On this background, there is a clear need for novel biomarkers that might improve the clinician's ability to manage optimally this often devastating condition by identifying those at high, and indeed those at low,[5] risk and/or guiding therapy.[6]

Current therapies target the primarily hormonal regulatory pathways such as the renin–angiotensin–aldosterone, and adrenergic and natriuretic peptide axes. At present, much energy is being directed into the investigation of molecular targets, such as regulatory RNAs at early and late steps in the development of, and response to, HF. For example, a positive therapeutic response to cardiac resynchronization therapy has been shown to be associated with specific changes in plasma microRNAs (miRNAs).[7] Most recently, another non-coding RNA species, namely circular RNAs (circRNAs) has been described. Here, we collect the current knowledge with regard to circRNAs as novel biomarkers and possible therapeutic targets in HF.

The RNA family

The 'Central Dogma of Molecular Biology' (Francis Crick, 1958) explicates that DNA material constituting our chromosomes is

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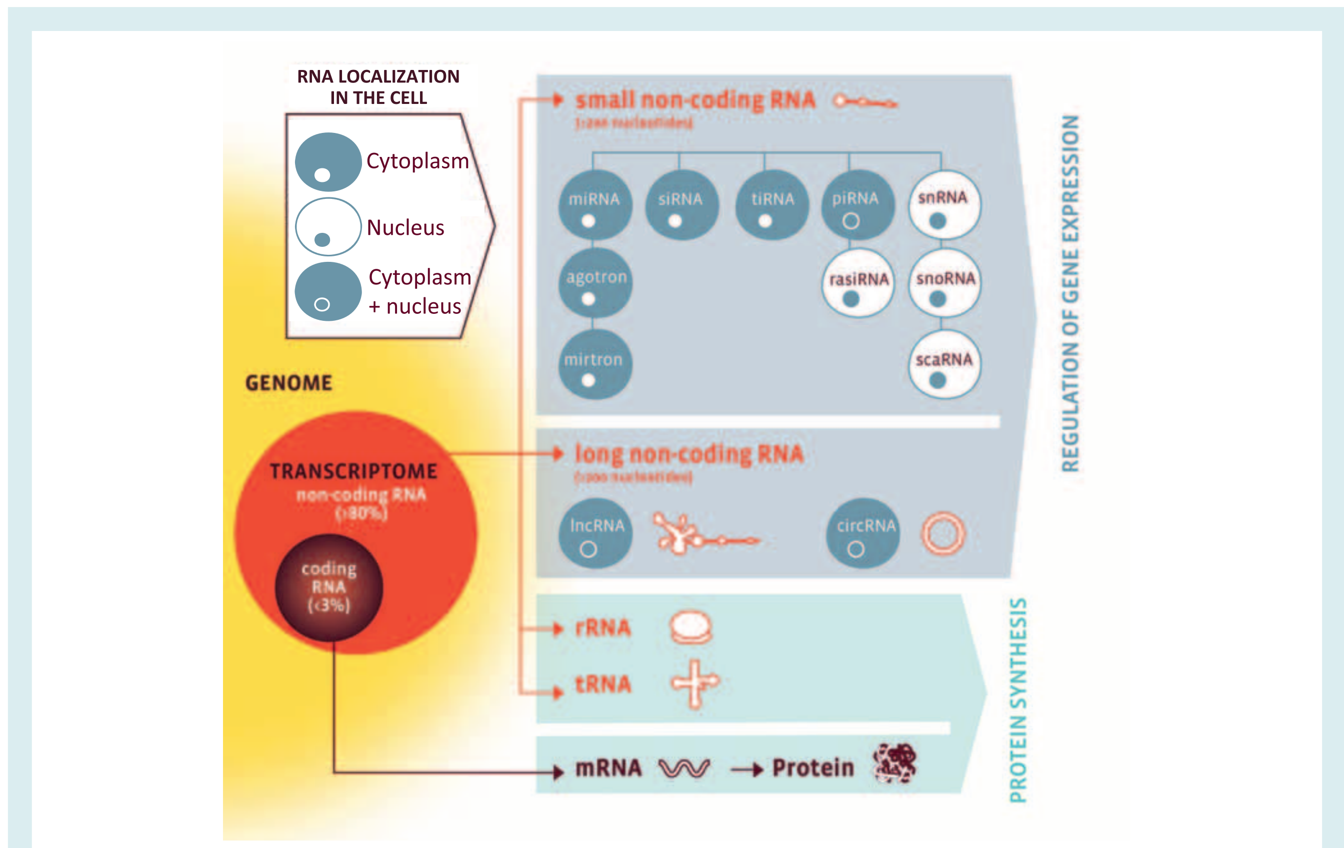

Figure 1 The RNA family. Different classes of RNAs can be distinguished by their protein-coding potential (coding or non-coding), their size (small or long), their function (protein synthesis, regulation of gene expression), and their subcellular localization (nuclear and cytoplasmic). Nuclear RNAs are shown in a white cell with blue nucleus, cytoplasmic RNAs in a blue cell with white nucleus, and RNAs present in both the nucleus and the cytoplasm are shown in a blue cell with blue nucleus. circRNA, circular RNA; lncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA; piRNA, piwi-interacting RNA; rasiRNA, repeat-associated small interfering RNA; rRNA, ribosomal RNA; scaRNA, small cajal body-specific RNA; siRNA, small interfering RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; tiRNA, tRNA-derived stress-induced small RNA; tRNA, transfer RNA.

transcribed into RNA molecules that are subsequently translated into proteins. This more than 50-year-old classic view has been challenged by the discovery that only 2% of the human genome encodes proteins and a large portion (~80%) is effectively transcribed into non-coding RNA molecules.[8] This finding implied that the vast majority of RNA species can be considered as non-protein-coding RNAs, in opposition to the well-known protein-coding RNAs or messenger RNAs (mRNAs). Together with intronic regions and repeated sequences, non-protein-coding regions of our genome have been for long considered as 'junk' DNA.[9] The 2000s have seen the emergence of a wealth of investigations dedicated to the 'Dark Side of the Genome',[10] and it appeared that non-protein-coding RNAs, also termed non-coding RNAs (ncRNAs), possess a much more critical regulatory role than previously thought.

Multiple types of RNAs have been described and can be classified according to their protein-coding potential, their subcellular localization, their size, and their mechanism of action (*Figure 1*). Unlike mRNAs, ncRNAs lack a protein-coding potential. Some ncRNAs such as miRNAs are found in the cytoplasmic compartment while circRNAs can be located both in the cytoplasm and the nucleus. CircRNAs, as well as long non-coding RNAs (lncRNAs), are classified as long, in opposition to small, non-coding RNAs. Furthermore, circRNAs, like lncRNAs, have been classified based on their orientation compared to their closest protein coding gene (sense, antisense, overlapping, etc.). Detailed descriptions of the mechanisms of action of major types of ncRNAs are available from recent reviews.[11–14] While the molecular mechanisms leading to regulation of HF development and progression by miRNAs and lncRNAs have attracted ample attention over the past decade and start to be rather well understood, the mechanisms through which circRNAs regulate gene expression and HF development are still poorly characterized.

Interestingly, circRNAs have been detected in the bloodstream,[15,16] raising the possibility that they might constitute a reservoir of novel biomarkers. Thus, due to their postulated ability to regulate gene expression and their presence in the circulation, circRNAs appeared as potential therapeutic and diagnostic tools for an improved health care of HF patients.

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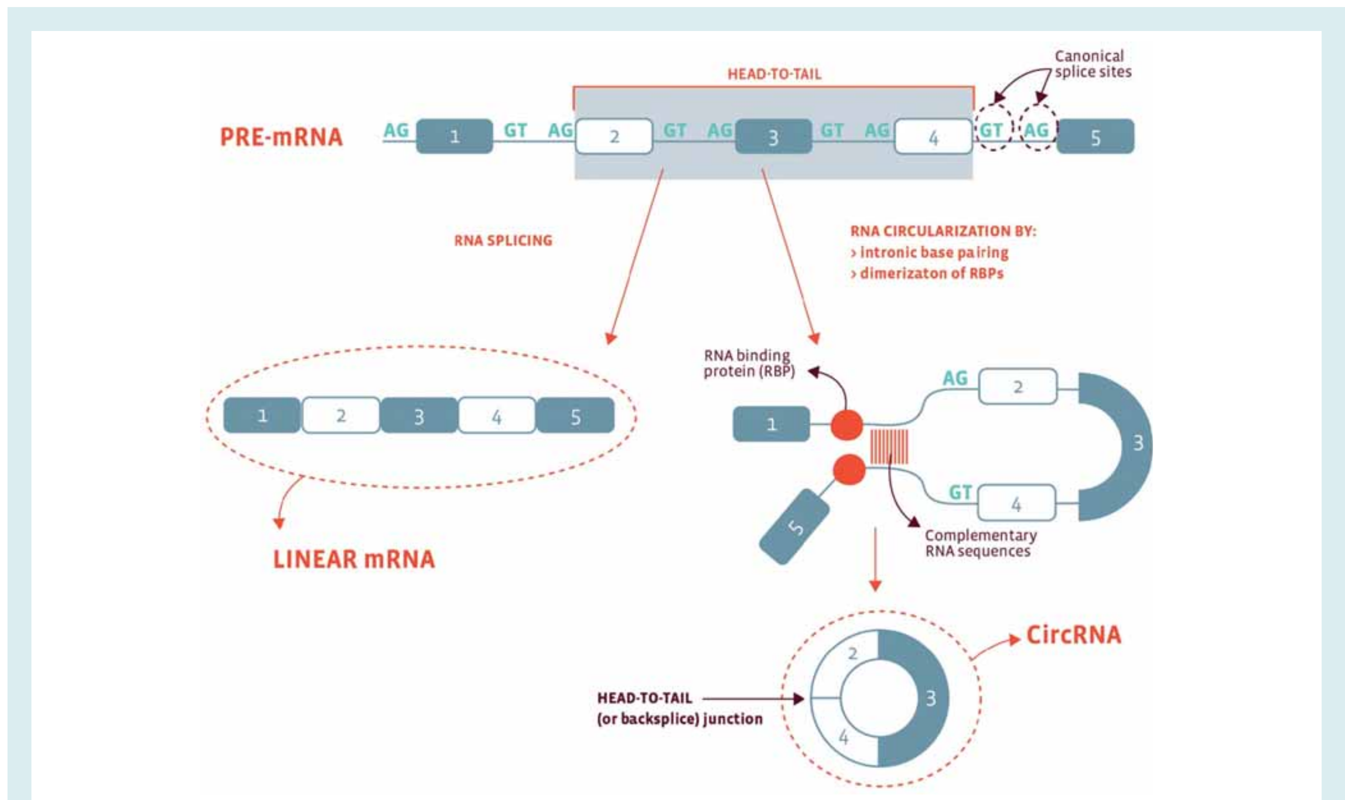

Figure 2 Circular RNA biogenesis. Circular RNAs are generated from pre-messenger RNA (pre-mRNA) by a back-splicing event of two exons. In this illustration, exon 4 associates with the upstream exon 2, instead of exon 5, as observed in linear splicing. This leads to a single-stranded RNA loop with a unique exon–exon junction of exon 2 and 4, which is not observed in the linear messenger RNA. It is believed that back splicing makes use of the canonical splicing machinery and canonical splice sites bracketing the exons. Factors that bring two introns in close proximity favour circularization. As such, the presence of inverted repeat sequences in introns can result in intronic base pairing, or RNA-binding proteins that interact with specific sequences in introns potentially dimerize and bring circularized exons closer together.

Mechanistic aspects of circular RNA biogenesis

CircRNAs represent a heterogeneous group of transcripts. A few databases have been generated that can be used to study circRNAs,[12] but information is still in its infancy. Recently, a catalogue of more than 32 000 human exonic circRNAs named circRNADb has been established.[17]

As other ncRNAs, circRNAs are detectable in many species starting from archaea and fungi to plants and animals. Their size ranges from 100 nucleotides to several kilobases and they harbour single or multiple exons.[13] While circRNAs mostly comprise protein-coding exons, some circRNAs contain both exons and introns, and others are derived from untranslated regions (5′ or 3′ UTRs) or introns of mRNAs or ncRNAs (e.g. lncRNAs).[18,19] The number of exons or introns included in a circRNA can vary greatly, ranging from one to dozens of exons. Most circRNAs contain two to three exons.[20] Some genes produce many different circRNAs derived from multiple exons or introns. The two genes that express the highest number of different circRNAs in the human heart are titin and the ryanodine receptor 2 (RYR2), which produce over 50 different circRNAs.[21,22] In general, circRNAs are expressed at low levels, but some are present at higher copy numbers than their linear counterparts.[23,24]

The biogenesis of circRNAs is linked to pre-mRNA splicing,[25,26] a process carried out by a sophisticated protein complex called the spliceosome that catalyses the removal of introns and joins exons together. CircRNAs are formed by a back-splicing event in one or two exons (*Figure 2*). In this back-splicing event, an exon is not associated with an adjacent downstream exon as observed in linear splicing, but the particular exon is associated with an upstream exon (or in the case of a single-exon circRNA, with the 5′ region of the same exon). This leads to single-stranded RNA loops with a unique exon–exon junction not present in the linear mRNA, called the head-to-tail junction or back-splice junction. Unlike typical linear RNAs, these single-stranded circRNAs lack the typical 5′ cap and poly(A) tail.

Back-splicing requires that the two introns flanking the circularized exons are brought into close proximity (*Figure 2*). This can be accomplished by direct RNA base pairing of reverse complementary sequences across flanking introns of the circRNA. As such, introns flanking circularized exons have been found to be generally enriched for Alu repeats in inverted orientation.[27] Alternatively, binding sites for RNA binding proteins such as Quaking

and Muscleblind in the flanking introns can also enhance circRNA production. It has been shown that the insertion of artificial Quaking binding sites into introns is sufficient to produce circRNAs,[28] and it was proposed that by forming dimers, Quaking brings the circularized exons in close proximity, thereby stimulating circRNA production. Another option for circRNA biogenesis is the formation of an exon-containing lariat precursor.[29]

A recent study by Khan *et al.* provided further evidence that circRNA production is intimately connected to alternative splicing.[21] They showed that the RNA-binding protein Rbm20, which was previously shown to be associated with cardiomyopathy and titin splicing,[30] is crucial for the formation of a subset of circRNAs from the titin gene. Specifically, circRNAs were produced from regions within titin known to undergo extensive Rbm20-mediated alternative splicing, and in Rbm20 knockout mice these titin-derived circRNAs were no longer produced. The authors proposed the concept that the cardiac splice factor Rbm20, by excluding specific exons from the pre-mRNA, provides a substrate to form circRNAs. This is in line with other studies that demonstrated that the more an exon is circularized, the less it is represented in the linearly processed mRNA.[25,26,31] Thus, the biogenesis of circRNAs appears as yet another pathway regulating the expression of protein-coding genes.

Function of circular RNAs in the heart

The function of naturally occurring or disease-regulated circRNAs in the heart is only known for a few circRNAs that have been studied so far (*Table 1*). The first circRNA that was described to be functional in the heart was termed heart-related circRNA (HRCR).[32] The authors showed that HRCR is normally present in mouse hearts and is repressed in hypertrophic and failing hearts. Biologically, HRCR binds and thereby sequesters miR-223, a miRNA that causes cardiac hypertrophy via inhibition of the protein ARC (apoptosis inhibitor with CARD domain). Overexpression of HRCR in an isoproterenol-induced hypertrophy mouse model inhibited hypertrophy, which the authors attributed to inhibition of miR-223. As it has been described for several other circRNAs, HRCR seems to function as a miRNA sponge, but whether this is a general mechanism for circRNA function remains to be confirmed.

Previously identified as a circular miRNA sponge for miR-7 in the brain,[33] CDR1AS was found to be induced in the heart after acute myocardial infarction (AMI) in mice.[34] CDR1AS was shown to be pro-apoptotic *in vitro*, consistently with the anti-apoptotic role of miR-7. More importantly, overexpression of CDR1AS in mouse hearts resulted in larger infarct sizes after AMI, which was prevented by simultaneous overexpression of miR-7. While these results are promising, the proof that the overexpressed CDR1AS is effectively expressed as a circRNA is lacking and the reliability of the unconventional *in vivo* transfection method used to achieve overexpression in these experiments needs to be demonstrated.

The only other circRNA that regulates cardiac function described to date is circ-Foxo3, which is increased in aged hearts.[35] Ectopic expression of this Foxo3-derived circRNA induced senescence in fibroblasts *in vitro* and silencing of circ-Foxo3 *in vivo* reduced doxorubicin-induced cardiomyopathy in mice. Functionally, circ-Foxo3 binds to several proteins known to be involved in cellular stress response, including inhibitor of DNA binding 1, E2F transcription factor 1, hypoxia-inducible factor 1α, and focal adhesion kinase, resulting in cytoplasmic sequestration of these proteins. Whether circ-Foxo3 contributes to cardiac ageing *in vivo* remains to be established.

CircRNAs in the vasculature that could potentially contribute to HF have also been described, most notably cZNF292.[36] cZNF292 is expressed in the endothelium and induced by hypoxia, so that one could envision that cZNF292 may be increased in the hypoxic myocardium. Experimental reduction of cZNF292 levels reduced endothelial function *in vitro*, suggesting that the increased levels of cZNF292 in hypoxic endothelial cells contribute to endothelial function and that this circRNA is involved in the regulation of endothelial cell function in the hypoxic myocardium.

Regulation of circular RNAs in the failing heart

The failing heart exhibits profound changes in transcriptional regulation, which may contribute to disease progression or constitute protective mechanisms. The identification and quantitative analysis of deregulated RNA molecules has proved to be a valid concept to define possible targets with biological significance and promise for therapeutic interventions. One of the most prominent transcriptional patterns of the failing myocardium is the re-expression of so-called fetal genes and gene isoforms such as beta myosin heavy chain, atrial natriuretic factor, and the titin isoform N2BA.[37] The fetal gene expression programme can be assessed through a comparison of gene expression in neonatal vs. adult hearts. Interestingly, many circRNAs show a strong regulation in this comparison, with the overall tendency being towards higher circRNA levels in neonatal myocardium.[22] Likewise, a tendency towards increased circRNA expression can be observed in failing human and mouse hearts, albeit to a lower extent. Due to this overall low extent of regulation, which might be partly attributed to the long half-lives of circRNAs, only a small number of statistically backed, strongly expressed ($\geq$3 back-splice-spanning reads) and differentially regulated circRNAs were so far reported in human HF.[21,22] Other than through transcriptional regulation (which also applies for other RNA species), the formation of circRNAs can be regulated directly during splicing, whereby exons that form the circRNA are no longer present in the mRNA of the respective gene (see previous section 'Function of circular RNAs in the heart' and Ashwal-Fluss *et al.*[25]). In this way, alterations in circRNA abundance in the failing heart may be caused by the differential activity of splicing factors as described for titin.[21]

Biomarker value of circular RNAs

Assessment of plasma levels of a natriuretic peptide (e.g. *N*-terminal pro brain natriuretic peptide, NT-proBNP) is part of the laboratory tests recommended by current guidelines for the

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Table 1 Circular RNAs in the cardiovascular system

CircRNA	CircBase ID	Host gene	Chromosomal location (hg19)	Predicted length (nucl)	Regulation	Function	Mechanism of action	Reference
HRCR	mmu_circ_0000254	Pwwp2a	chr11: 43518035-43518979	944	Repressed in hypertrophic and failing hearts	Inhibits cardiac hypertrophy and heart failure	Endogenous sponge for miR-223	(32)
CDR1AS	hsa_circ_0001946	CDR1	chrX: 139865339-139866824	1485	Induced in the infarcted hearts	Pro-apoptotic	Endogenous sponge for miR-7	(34)
Circ-Foxo3	hsa_circ_0006404	Foxo3	chr6: 108984657-108986092	1435	Up-regulated in aged hearts	Increases cellular senescence	Retains ageing factors in the cytoplasm	(35)
cZNF292	hsa_circ_0004383	ZNF292	Chr6: 87920168-87928449	8281	Induced by hypoxia in the endothelium	Pro-angiogenic	Induces tube formation and endothelial cells sprouting	(36)
cTTN1	-	TTN	chr2:179542852-179585929	11178	Down-regulated in dilated hearts	Unknown	Formation is Rbm20 dependent	(21)
cTTN2	-	TTN	chr2:179542852-179580501	8355	Down-regulated in dilated hearts	Unknown	Formation is Rbm20 dependent	(21)
cTTN4	-	TTN	chr2:179554540-179580501	8898	Down-regulated in dilated hearts	Unknown	Formation is Rbm20 dependent	(21)
cTTN5	-	TTN	chr2:179539041-179580501	11466	Down-regulated in dilated hearts	Unknown	Formation is Rbm20 dependent	(21)
MICRA	hsa_circ_0000615	ZNF609	chr15: 64791491-64792365	874	Down-regulated in blood cells of patients with left ventricular dysfunction	Unknown	Unknown	(15)

circRNA, circular RNA; HRCR, heart-related circRNA; MICRA, myocardial infarction-associated circRNA; nucl, nucleotides.

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diagnosis of acute HF.[1] Although the benefit provided by biomarkers for the management of HF patients is widely acknowledged, the use of NT-proBNP-guided therapy of HF patients suffers from limitations and might not be recommended in all patients.[6,38,39] Although several biomarkers have been associated with outcome after acute HF, their benefit for the patient has not yet been demonstrated and none of them are recommended for prognostication purposes.[1] In addition, using BNP to identify patients at risk of developing HF after AMI is strongly limited by fluctuations of circulating levels of BNP, especially after an anterior infarct.[40] Consistently, BNP was a poor indicator of prognosis in different cohorts of AMI patients.[15,41] Therefore, novel HF biomarkers are needed.

Members of the RNA family have shown some promise as novel HF biomarkers, especially miRNAs.[42,43] One of the most widely studied was miR-423-5p, the circulating levels of which have been shown to be associated with outcome of HF patients.[44–47] Interestingly, circulating levels of miR-423-5p increased in response to hypertension-induced HF, suggesting that this miRNA (along with others) might be used to monitor treatment efficacy.[48] A recent investigation of the transcardiac gradient of miRNAs in the failing heart supports the potential of miRNAs as biomarkers as well as therapeutic targets of HF.[49]

Long non-coding RNAs, constituting another arm of the non-coding branch of the RNA family (*Figure 1*), are regulated after AMI[50] and in the failing heart,[51,52] and are involved in cardiac remodelling.[53,54] Yet, their biomarker potential appears to be limited by their low stability in plasma and serum samples. Nevertheless, a mitochondrial lncRNA detected in the plasma and named LIPCAR (long intergenic non-coding RNA predicting cardiac remodelling) predicted cardiac remodelling after AMI and death in HF patients.[55] In another investigation, two lncRNAs detected in the blood—cyclin-dependent kinase inhibitor 2B antisense RNA 1 (ANRIL) and potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ1OT1)—improved outcome prediction after AMI.[56] Whether the association between ANRIL and outcome after AMI is related to the correlation between linear and circular forms of ANRIL and atherosclerosis risk[57] is so far unknown.

Circular RNAs have a great biomarker potential for the following reasons. First, they are extraordinarily stable due to the lack of exposed terminal ends which are susceptible to degradation by exoribonucleases (RNases), and certain RNA folding determined by their structure. Half-lives of some circRNAs exceed 48 h[16] (*Figure 3*). Second, deep RNA-sequencing has identified several hundreds to thousands of cell-specific circRNA loci in human and mouse cells and tissues.[24] Third, they are present in whole blood, plasma and extracellular vesicles.[15,16,58] Fourth, their expression is regulated during cardiac development[22] and in the failing heart.[21,59] Fifth, it has been estimated that circRNAs account for 1% of poly(A) RNAs[24] and thus represent a non-negligible reservoir of potential biomarkers. Sixth, they circulate in large amounts in the blood.[60] Most of these good reasons to search for novel biomarkers among circRNAs also apply to miRNAs.[61] Yet, not much was known about the potential of circRNAs as biomarkers of cardiac disease until it was recently shown that the expression level in blood cells of a circRNA called MICRA (myocardial infarction-associated circular RNA) predicted left ventricular remodelling after AMI.[15] Importantly, the predictive value of MICRA was confirmed in two independent cohorts totalling more than 600 patients and it improved prognostication by existing markers. This study strengthened the concept that blood cells constitute a source of novel circRNAs with potential biomarker value.[62]

Several high-throughput approaches such as RNA sequencing, preferably preceded by a treatment of RNA with RNase R to enrich for circRNAs, can be used to investigate the regulation of circRNAs in biomarker studies. Before being considered as a potential biomarker, a novel circRNA must be subjected to extensive validation of its circularity, using digestion with RNase R, assessment by polymerase chain reaction (PCR) using divergent primers, and Sanger sequencing of the junction region. Quantitative PCR remains the most widely used technique to assess the expression level of circRNAs.

Although significant technological developments have been achieved during the last decade to allow the emergence of molecular diagnostic assays,[62] additional work is needed to overcome the technical hurdles to implement circRNAs as biomarkers.

Conclusions and future directions

The knowledge of the expression patterns of circRNAs, their regulation in disease states, pathophysiological role, and potential use for therapeutic or diagnostic purposes is still at an early stage. To date, only a handful of circRNAs have been shown to be involved in cardiac function. Whether other circRNAs contribute to HF remains to be established. One can imagine that circRNAs in different cell types involved in HF, such as cardiomyocytes, fibroblasts, and inflammatory cells may contribute to the pathogenesis of HF and thus may constitute potential therapeutic targets.

Similarly to other better characterized members of the RNA family such as miRNAs and lncRNAs, circRNAs appear to have the potential to aid in personalizing the health care of HF patients. Yet, extensive discovery and validation studies are required before circRNAs can be considered as clinically valuable HF biomarkers.

The following recommendations for future research may help deepen our knowledge of the role of circRNAs in the failing heart and in addressing their value as biomarkers:

- An in-depth characterization of the molecular mechanisms leading to the biogenesis of circRNAs is warranted.
- A profiling of the temporal-, cell- and tissue-specific expression of circRNAs in the healthy and failing heart would represent an important source of information.
- A better characterization of the protein-coding potential of circRNAs is necessary.
- A functional characterization of circRNAs is needed, notably regarding their role as miRNA sponges and epigenetic regulators.
- A detailed knowledge of the role of circRNAs in HF development would ease the identification of potential therapeutic targets.
- A reflection around the possible therapeutic approaches based on targeting circRNAs is required. A characterization of the

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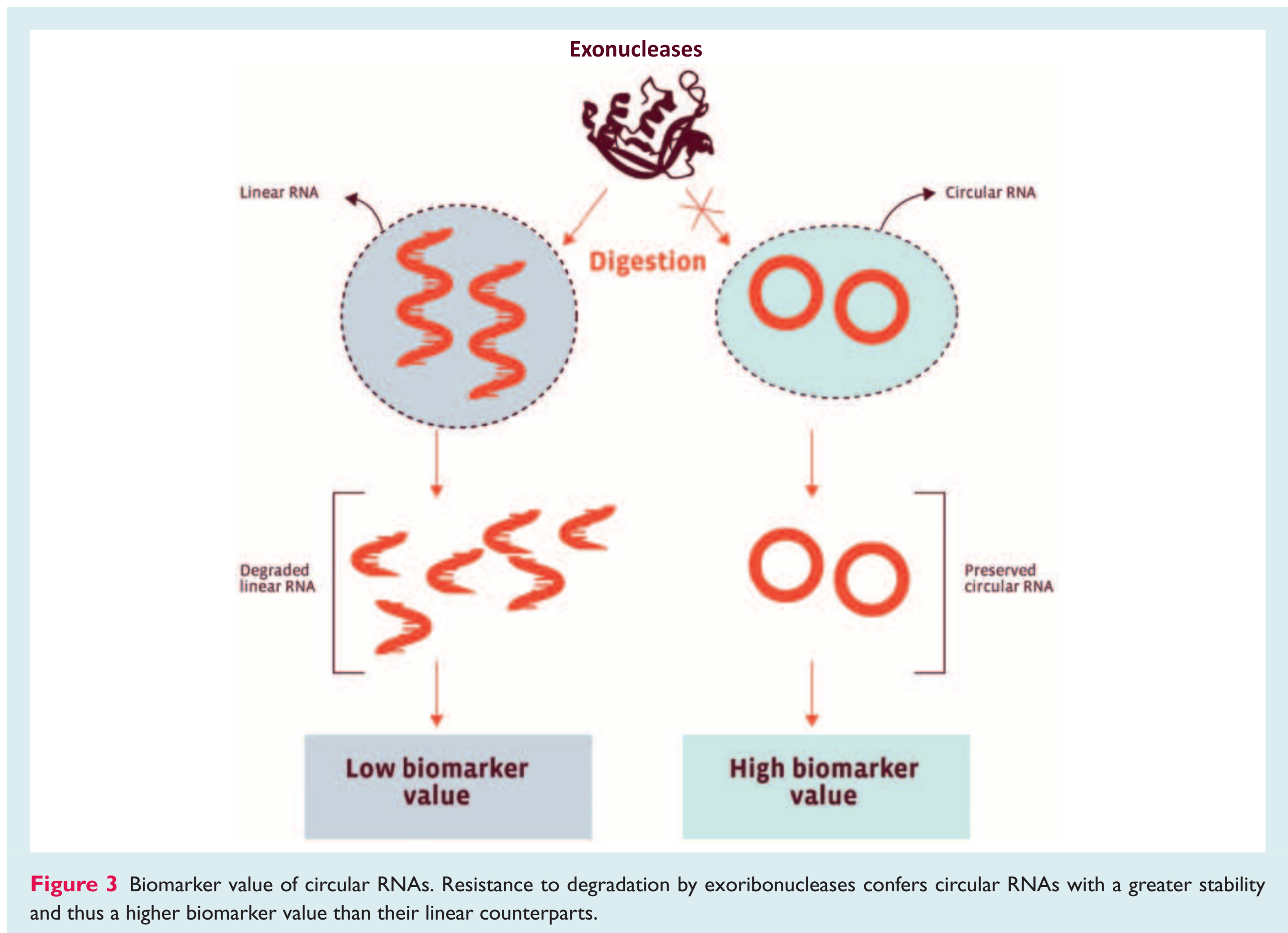

Figure 3 Biomarker value of circular RNAs. Resistance to degradation by exoribonucleases confers circular RNAs with a greater stability and thus a higher biomarker value than their linear counterparts.

full spectrum of circRNAs circulating in the bloodstream would help in selecting circRNAs for future biomarker studies.

- Large-scale biomarker studies are required to validate candidate circRNAs as HF biomarkers.
- Standardization of the assessment of circRNAs and development of clinically applicable diagnostic assays represent challenges for future work.

Overall, recent findings motivate the search for novel therapeutic targets and biomarkers among circRNAs to aid in moving personalized health care of HF patients a step forward. In modern medicine, the clinician scientist relies upon the laboratory scientist to advance our ability to personalize medicine; circular RNAs may represent just such an opportunity.

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