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Review Article

Role of microRNAs in arrhythmogenic cardiomyopathy: translation as biomarkers into clinical practice

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ABSTRACT

Arrhythmogenic cardiomyopathy is a rare inherited entity, characterized by a progressive fibro-fatty replacement of the myocardium. It leads to malignant arrhythmias and a high risk of sudden cardiac death. Incomplete penetrance and variable expressivity are hallmarks of this arrhythmogenic cardiac disease, where the first manifestation may be syncope and sudden cardiac death, often triggered by physical exercise. Early identification of individuals at risk is crucial to adopt protective and ideally personalized measures to prevent lethal episodes. The genetic analysis identifies deleterious rare variants in nearly 70% of cases, mostly in genes encoding proteins of the desmosome. However, other factors may modulate the phenotype onset and outcome of disease, such as microRNAs. These small noncoding RNAs play a key role in gene expression regulation and the network of cellular processes. In recent years, data focused on the role of microRNAs as potential biomarkers in arrhythmogenic cardiomyopathy have progressively increased. A better understanding of the functions and interactions of microRNAs will likely have clinical implications. Herein, we propose an exhaustive review of the literature regarding these noncoding RNAs, their versatile mechanisms of gene regulation and present novel targets in arrhythmogenic cardiomyopathy.

Abbreviations: ACM, arrhythmogenic cardiomyopathy; BIN1, bridging integrator 1; CDH2, cadherin 2; cTNI, troponin; CTNNA3, T-catenin; DES, desmin; DSG2, desmoglein-2; DSP, desmoplakin; FLNC, filamin C; GAL-3, galetin-3; HSP70, heat shock protein 70; IVT, idiopathic ventricular tachycardia; JUP, plakoglobin; LMNA, lamin A/C; miRNA, microRNA; mRNA, messenger RNA; ncRNA, noncoding RNA; NGS, next-generation sequencing; PLN, phospholamban; PPKP2, plakophilin-2; RV, right ventricle; SCD, sudden cardiac death; SCN5A, sodium voltage-gated channel alpha subunit 5; TGFβ, transforming growth factor β; TMEM43, transmembrane protein 43; TTN, titin; VF, ventricular fibrillation; VT, ventricular tachycardia; VUS, variant unknown significance

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ARRHYTHMOGENIC CARDIOMYOPATHY

Arrhythmogenic cardiomyopathy (ACM) includes the classical abnormality of the right ventricle (RV) in which the myocardium is progressively substituted by fibro-fatty tissue,[1] named firstly arrhythmogenic right ventricular dysplasia and later arrhythmogenic right ventricular cardiomyopathy. In recent years, solely left ventricular and biventricular phenotypes have been also reported. This progressive tissue substitution leads to palpitations, syncope and/or cardiac arrest, secondary to ventricular tachycardia (VT) or fibrillation (VF).[2] Sudden cardiac death (SCD) may be the first manifestation of the disease, occurring even in the absence of an evident structural alteration of the myocardium, as a consequence of electrical disruptions that lead to malignant arrhythmia.[3] This occurs in the early stages of the disease due to protein conformational changes without a high degree of fibro-fatty replacement of cardiomyocytes. SCD episodes usually occur in young men, with physical exercise being the main trigger of the lethal arrhythmias in genetically predisposed hearts.[4]

ACM is a rare arrhythmogenic entity with an estimated prevalence between 1:2000 and 1: 5000 (OMIM #107970; ORPHA247), depending on the population. ACM is an inherited disorder, characterized by incomplete penetrance and variable phenotype expressivity.[5] The pathophysiological mechanisms that underpin this diverse and pleiotropic disease remain poorly characterized.[6] Therefore, relatives of a diagnosed patient who carry the same deleterious variant may be at risk of malignant arrhythmias, despite being asymptomatic. ACM affects adolescents infrequently and children rarely, despite clinical assessment.[7] In recent years, thanks to the continuous improvement of genetic technologies, several rare variants in different genes, mainly encoding desmosomal proteins, have been identified.[8] Genetic analysis is often part of clinical diagnosis and it is highly recommended in relatives of an ACM diagnosed family member.[9] The main gene responsible for almost 40% of ACM cases is the *PKP2* gene, encoding the plakophilin-2 protein.[2] Genetic studies of other components of the cardiac desmosome have been associated with nearly 20% of ACM patients, specifically those genes encoding: desmocollin-2 (*DSC2*), desmoplakin (*DSP*), desmoglein-2 (*DSG2*), and plakoglobin *(JUP)*.[10] In addition, other minority genes have been also reported as a potential cause of ACM, such as alpha-actinin-2 (*ACTN2*), cadherin 2 *(CDH2),* T-catenin *(CTNNA3),* desmin (*DES),* filamin C *(FLNC),* LIM domain binding protein 20 (*LDB3*), lamin A/C *(LMNA),* phospholamban *(PLN*), RNA binding motif protein 20 *(RBM20),* sodium voltage-gated channel alpha subunit 5 *(SCN5A),* transforming growth factor-β3 *(TFGB3),* tight junction protein 1 (*TJP1*), transmembrane protein 43 *(TMEM43),* and titin *(TTN)*.[11] Although ACM usually follows an autosomal dominant pattern of inheritance, recessive pattern phenotypes have also been reported in familial Carvajal syndrome (deleterious variant in *DSP)*,[12,13] Naxos disease (deleterious variant in plakoglobin -*JUP*-) and deleterious homozygous variants in *DSC2*[14] (Table 1).

Nowadays, a comprehensive genetic analysis of ACM-associated genes may unveil the genetic cause in almost 70% of families.[2] Despite improvement in genetic sequencing, a large part of identified rare variants remains without a conclusive deleterious role in ACM, mainly due to a lack of functional studies.[15] Therefore, a reanalysis is necessary, despite no time frame having been established so far. In order to determine the periodicity of the reanalysis in a previous study by our group, we suggested 5 years as the maximum time for reanalysis of rare variants, mainly those classified with inconclusive/unknown significance (known as VUS) should be performed before clinical translation of genetic results.[16] This is a key point, due to the fact that the diagnosis of ACM can be challenging and current guidelines consider genetic data for differential diagnosis. Thus, the identification of a definitely deleterious rare variant may modify the final diagnosis, even though no specific clinical treatment targeting the mutation or the causative gene of the pathology is known yet.

PATHOPHYSIOLOGICAL MECHANISMS

The molecular mechanisms by which desmosome deleterious alterations lead to pathological hallmarks of ACM, such as fibrosis, cardiomyocyte loss, adipogenesis, inflammation, and arrhythmogenesis are still poorly defined to date. Probable pathophysiological mechanisms include loss of mechanical integrity at cell-cell junctions, altered signaling pathways at intercalated discs, disruption of ion channels and gap junctions, and aberrant protein trafficking (Fig 1). In some of them, miRNA have already described to be involved.

Cardiomyocyte loss

Loss of cardiomyocytes is an important feature in the ACM progression. Often, patients present alterations in desmosomal proteins and, therefore, these cell junctions are weak and may have less mechanical strength. This might provoke cell injuries caused by heart contraction stress, resulting in cellular death.[17] This damage may be aggravated by

Table 1
Genes and pattern of inheritance associated with ACM

Gene	Protein	Inheritance	Cell structure	Anatomic affection
ACTN2	Alpha-actinin-2	AD	Cytoskeleton	LV
CDH2	Cadherin 2	AD	Cytoskeleton	RV/BiV
CTNNA3	T-catenin	AD	Cytoskeleton	RV/BiV
DES	Desmin	AD	Cytoskeleton	LV/BiV
DSC2	Desmocollin-2	AD / AR	Desmosome	RV/BiV
DSG2	Desmoglein-2	AD	Desmosome	RV/LV/BiV
DSP	Desmoplakin	AD / AR (Carvajal)	Desmosome	LV/BiV
FLNC	Filamin C	AD	Cytoskeleton	LV
JUP	Plakoglobin	AD / AR (Naxos)	Cytoskeleton	RV/BiV
LDB3	LIM domain binding protein 20	AD	Cytoskeleton	RV
LMNA	Lamin A/C	AD	Cytoskeleton	LV/BiV
PLN	Phospholamban	AD	Calcium homeostasis	LV/BiV
PKP2	Plakophilin-2	AD	Desmosome	RV/BiV
RBM20	RNA binding motif protein 20	AD	Cytoskeleton	LV
SCN5A	Sodium voltage-gated channel alpha subunit 5	AD	Sodium transport	RV/LV/BiV
TGFB3	Transforming growth factor-β3	AD	Cytoskeleton	RV
TJP1	Tight junction protein	AD	Cytoskeleton	RV/BiV
TMEM43	Transmembrane protein 43	AD	Cytoskeleton	RV/BiV
TTN	Titin	AD	Cytoskeleton	RV/LV/BiV

Abbreviations: AD, autosomic dominant; *AR*, autosomic recessive; *BiV*, biventricular involvement; *LV*, left ventricular involvement; *RV*, right ventricular involvement.

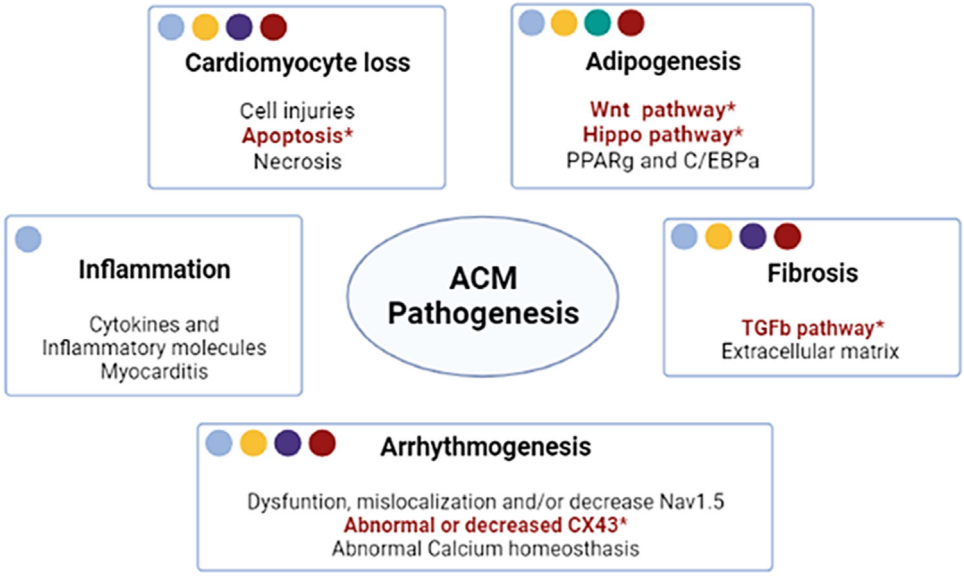

Fig 1. Schematic representation of basic pathophysiological mechanisms involved in ACM. Circles indicate triggers and/or other factors involved: blue, genetic mutations; yellow, physical activity; green, androgens; purple, mitochondrial dysfunction and red, miRNA. *Indicates molecular pathways associated to altered miRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increasing the mechanical stress in the heart during exercise. It is known that in normal conditions, physical activity promotes an upregulation of *JUP* and *JUP*-associated proteins, as well as gap junction (GJ) proteins to face the mechanical stress.[18] Hence, cardiomyocytes with desmosomal pathogenic alterations are incapable of responding to the stretch pressure caused by a higher level of physical activity, thus altering their mechanical properties and leading to myocytes death.[19] Another mechanism contributing to cardiomyocyte loss is cardiomyocyte apoptosis; aberrant nuclear localization of plakoglobin in cardiomyocytes acts as an antagonist to Wnt-ß-catenin signaling, leading to apoptosis in an animal model of ACM.[20] Moreover, the increase of glycogen synthase kinase 3β (GSK-3β) also contributes to apoptosis by promoting catenin-β1 degradation and impairing the Wnt/ß-catenin pathway.[21] Finally, activation of the EP300-TP53 pathway, known to suppress the Hippo and canonical Wnt pathways, was detected in right and left ventricular endomyocardial biopsy from ACM patients, and was associated with increased apoptosis, suggesting a pathogenic role during ACM.[22]

Fibrosis

Cardiac fibrosis is a common pathological change associated with cardiac injuries and disease. Since the heart is not able to regenerate its muscular tissue, cardiomyocytes are substituted by cardiac fibroblasts, which proliferate and differentiate in myofibroblasts, resulting in excessive extracellular matrix (ECM) deposition and consequent mechanical stiffness.[23] In ACM patients, fibrosis is promoted by complex interactions between cytokines, growth factors and hormones.[17] The transforming growth factor beta (TGF-β) is a pivotal cytokine/growth factor that contributes to cardiac fibrosis by phosphorylating and activating the receptor-associated SMAD, that triggers the expression of profibrotic genes such as ECM proteins and inhibitors of matrix metalloproteinases (MMP).[24] Li et al[25] showed increased TGFβ1 expression levels and activation of SMAD2 signaling in an ACM mouse model associated with a rare variant in the *JUP* gene. Moreover, the β-adrenergic system could also regulate the extracellular matrix protein turnover. Norepinephrine could trigger the expression of tissue inhibitor of metalloproteinases 1 (TIMP-1), by decreasing metalloproteinase 2 MMP-2/TIMP-1 ratio.[26,27]

Mechanical stress caused by a high level of physical activity could increase plasmatic levels of TGFβ1.[26] On the other hand, the noncanonical TGFβ pathway also plays a role in cardiac fibrosis by activation of mitogen-activated protein kinase (MAPK) signaling and generation of reactive oxygen species.[28] A knockdown of *pkp2* in a cell model presented an upregulation of fibrotic genes via noncanonical TGFβ−MAPK signalling.[29] All these data suggest that both canonical and noncanonical TGFβ signaling can have a role in inducing myocardial fibrosis in ACM. Fibrosis is magnified by a positive loop promoted by changes in extracellular matrix composition that vary tissue properties by increasing its rigidity. Subsequently, tissue stiffness promotes the differentiation of myofibroblasts that produce and release collagen, thus further increasing the stiffness of the tissue.[26]

Inflammation

Inflammatory infiltrates are frequently observed in ACM biopsy samples, commonly in fibrotic areas.[30-32] Myocarditis, mainly caused by cardiotropic viruses' infection, may herald the onset of ACM and contributes to its progression.[33] Differential diagnosis between myocarditis and ACM is still challenging and difficult to distinguish in some patients who show an overlap of clinical features, such as desmosome disruption, inflammatory infiltrates and the presence of viral genomic material.[34] Some authors suggest that ACM could be a form of myocarditis due to this clinical overlap.[31] Moreover, this hypothesis has been published as a case report that presents a patient who died of myocarditis but also fit in ACM diagnosis. Recently, some studies state that nearly 10% of cases diagnosed with myocarditis carried deleterious variants in genes associated with ACM.[35-39] It has been suggested that desmosomal dysfunction stimulates the production of inflammatory molecules, but it is not clear whether myocardial inflammation is a primary cause of ACM or just a secondary response to cardiomyocyte death.[40]

Adipogenesis

Adipogenesis is a common feature of ACM that consists of the infiltration of adipocytes, in this case, into the heart tissue. The cellular source of these adipocytes and the molecular signals are still under discussion. Regarding the cellular origin of adipocytes, there are several candidates: cardiomyocytes, differentiated nonmyocytes, cardiac progenitor cells and circulating progenitor cells.[1] There are numerous studies in mouse models showing that second heart field progenitor cells are involved in the origin of adipocytes.[41-44] Moreover, another study

proved the ability of cardiomyocytes to transdifferentiate into adipocytes *in vitro*.[45] All these data contribute to elucidate the origin of adipocytes, but more studies are needed to reach a consensus about their source in ACM. On the other hand, molecular signals triggering adipogenesis are also under investigation. Several signaling pathways have been described to be involved in adipogenesis.[17] There is evidence that the downregulation of the Wnt pathway contributes to adipogenesis by triggering the differentiation of mesenchymal stem cells and preadipocytes into adipocytes.[46] In the absence of Wnt ligands, GSK-3β phosphorylates catenin-β1. The interaction between GSK-3β and catenin-β1 is stabilized by Axin, which serves as a negative regulator of the canonical Wnt pathway.[47,48] Also, in the presence of Wnt ligands, GSK-3β no longer phosphorylates catenin-β1, that accumulates in the cytoplasm and translocates into the nucleus where it co-activates the T-cell/lymphoid enhancer (TCF-LEF) transcription factors that will upregulate the expression of adipogenic genes.[49-51]

BIOMARKERS

Early diagnosis and risk stratification are crucial for identifying at-risk patients who need therapeutic intervention. The features of a biomarker include high specificity, accuracy, reproducibility, ease procurement and a low price. A biological marker needs to add value over the existing diagnostic tests, being useful for clinical decision-making. One of the main limitations of biomarkers related to ACM is their lack of specificity, not being useful in optimizing clinical decision-making in this population. Some of the proposed biomarkers, such as troponin I (cTNI) or heat shock protein 70 (HSP70), have not shown any disease-specificity. Concretely, cTNI has been associated with ACM, but this protein informs us more about a myocardial injury than disease-specificity, not leading to a certain CVD etiology. Thus, apoptosis and impairment of the intercalated discs may justify a release of this myocardial protein in ACM.[52] However, cTNI plasmatic concentration may be increased in several situations, such as extreme exercise, myocarditis or myocardial ischemia, among others. HSP70 is a chaperone which was described to be associated with damaged myocardial cells in ACM failing hearts compared to nonfailing hearts[53]; however, a high level of this protein has also been found in dilated and ischemic cardiomyopathies in response to tissue damage. Bridging integrator 1 (BIN1) is a protein that belongs to the BAR domain superfamily and organizes membrane structure and function. BIN1 is involved in cardiomyocyte calcium homeostasis and, when altered, leads to an excitation-contraction impairment. Hong et al[54] established a correlation between BIN1 and heart failure in a small cohort of ACM patients. Although BIN1 is not directly linked to cardiac desmosomes, reduced concentrations of this protein predicted ventricular arrhythmia events in this population. Nevertheless, this marker does not discriminate between early-stage ACM patients without heart failure and healthy subjects, furthermore, BIN1 leads to a pathologic cardiomyocyte alteration more than to contractile dysfunction.

Currently, some of the proposed circulating blood biomarkers remain potential specific markers for late phase ACM. Brain natriuretic peptide (BNP) has been described as an indicator of ventricular dysfunction in ACM patients, with a negative correlation with RV ejection fraction, helping to discriminate between idiopathic ventricular tachycardia (IVT) and ACM.[55] Recently, N-terminal fragment of BNP (NT-pro-BNP) was positively correlated with RV end-diastolic and end-systolic diameter, as well as negatively associated with RV ejection fraction assessed by cardiac magnetic resonance.[56,57] Moreover, NT-proBNP confirmed to be increased in patients with biventricular ACM.[58] The main flaw of these molecules is their low specificity.[59] Another circulating marker is interleukin-33 receptor ST2, associated with fibrosis and cardiac remodeling, which has been correlated to RV and/or left ventricular dysfunction and potential lethal arrhythmias in the ACM population.[60] Finally, Galectin-3 (GAL-3), an inflammatory protein related to fibrosis, was also found to be increased in plasma of ACM patients, thus correlating with more susceptibility to ventricular arrhythmia.[61] This marker has also been demonstrated to be elevated in heart failure cohorts at risk of sudden death due to VF or VT.[62]

Several studies have also identified increased levels of pro-inflammatory cytokines in the plasma and serum of ACM patients, thus suggesting them as candidate circulating biomarkers.[63,64] Likewise, C-reactive protein was detected at higher levels in the plasma of ACM patients immediately after an episode of ventricular arrhythmia, compared to patients with IVT, thus suggesting a correlation with arrhythmic events in ACM, unrelated to structural and/or functional anomalies of the heart.[65] However, the specificity of these circulating inflammatory mediators remains limited for ACM, since elevated cytokine levels have been described in several cardiovascular diseases.

NONCODING RNAS

The diagnosis of ACM is based on a scoring system with major and minor criteria.[66] However, an early identification of ACM is particularly difficult, taking into account the absence of a single clinical standard to reach the diagnosis, overlapping phenotypes with other cardiac diseases, incomplete penetrance and variable phenotype expressivity, as well as the genetic diagnosis limitations. Moreover, the lack of specific diagnosis tools, either imaging tests or circulating markers, leads to an urgent need for novel biomarkers in ACM. Steady progress in the discovery of new biomarkers and more sophisticated clinical applications offer promising possibilities for enhancing the care of patients.

In the last few years, researchers and clinicians have identified noncoding RNAs (ncRNAs) as useful biomarkers due to their presence in biological fluids, such as plasma and serum. ncRNA have a wide variety of types based on function and size (Fig 2, Table 2). Many cardiovascular diseases have shown an aberrant expression of ncRNAs in cardiac cells involved in pivotal regulatory roles in pathophysiological processes, and several reports have described the diagnostic potential of circulating ncRNAs in cardiovascular diseases.[67,68] Specific patterns of ncRNA expression have been related to certain cardiovascular disorders.[69] These molecules are of great value as diagnostic/prognostic biomarkers for heart failure and several cardiac diseases. Thus, ncRNAs are a group of genes without protein codification. Briefly, they can be divided into housekeeping ncRNAs, mainly tRNAs, rRNAs, and snRNAs, which play crucial roles in cellular processes. Others are regulatory ncRNAs, mainly miRNAs, small interfering RNAs (siRNAs) and long noncoding RNAs (lncRNAs), which are tissue-specific and work in response to various internal and environmental stimuli.

MiRNAs have emerged as main players in human physiological and pathologic processes, including cardiovascular phenotypes.[70] In recent

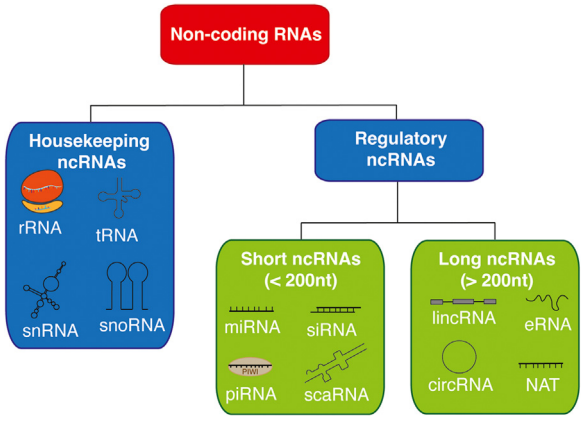

Fig 2. Classification of noncoding RNA. Noncoding RNA can be divided into 2 groups: housekeeping and regulatory ncRNA. The regulatory ncRNA can also be divided into 2 groups based on their size (long and short ncRNA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Main noncoding RNA (ncRNA) classes

Name	Acronym	Length (nt)	Description
Circular RNA	circRNA	≈100–1600	Covalently closed RNA rings; some have coding functions; potential gene regulators.
Small interfering	siRNA	20–25	Double-stranded RNAs similar to miR, operating through RNA interference (RNAi) pathway; promote mRNA degradation
Micro-RNA	miRNA/miR	21–24	Function in RNA silencing and post-transcriptional regulation of gene expression; may have an extracellular localization
Piwi-interacting RNA	piRNA	26–31	Epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells.
Small nuclear RNAs	snoRNAs	≈60–200	Guide chemical modifications of other RNAs (rRNA, tRNA, snRNA)
Small nuclear RNAs	snRNA/U-RNA	≈150	Function in the processing of pre-messenger RNA (hnRNA) in the nucleus; aid in the regulation of transcription factors; telomere maintenance
Natural antisense transcripts	NAT	>200	RNAs encoded within a cell that have transcript complementarity to other RNA transcripts; RNA interference (RNAi); alternative splicing, genomic imprinting; X-chromosome inactivation.
Enhancer RNAs	eRNA	50–2000	Transcriptional regulation in cis and in trans

Long noncoding RNAs are considered >200 nt in length. Short noncoding RNAs are considered <200 nt.

years, the study of miRNAs as potential biomarkers in ACM has progressively increased. This review provides an overview on the role of microRNAs (miRNAs) in ACM, focusing on the modulated biological pathways and their potential use as potential biomarkers; moreover, we discuss future clinical applications and limitations of this novel tool.

MicroRNAs

MiRNAs are single-stranded, small RNA comprising 19–25 nucleotides endogenously expressed, evolutionarily conserved in animals.[71] MiRNAs are involved in complex networks that allow interaction and control of the cellular phenotype. Most of the biological processes such as cell cycle control, proliferation, differentiation, inflammation, apoptosis, autophagy, or stress response are affected by miRNAs.[72] Their principal function is the regulation of gene expression through translational inhibition and/or promoting degradation of target messenger RNA (mRNA). They show a pleiotropic function as a single miRNA that can regulate different mRNAs, and each of the regulated protein-coding genes can be modulated by various miRNAs, thus allowing cooperation between miRNAs. Also, miRNAs themselves can be modulated by feedback mechanisms promoted by the protein products of mRNA targets.[73] The general mechanism of miRNA action is based on pairing the so called ''seed'' sequence (miRNA nt 2–8) with complementary sites of the target messenger RNA, mainly located in the 3′-untranslated region (3′UTR), but also in the coding region or in the 5′UTR The role of RNA structure at 5′ untranslated region in microRNA-mediated gene regulation.[74,75] A full or partial miRNA complementarity with the targeted mRNAs can determine the nature of target repression.[76] Complete base-pairing leads to mRNA endonucleolytic cleavage, while partial base-pairing results in the repression of mRNA translation through interference with the translational machinery (directed to polyribosomes in subcellular compartments, resulting in truncated protein) or by sequestering in cytoplasmic P-bodies. To date, over 2500 human mature miRNAs have been annotated in the miRBase (http://www. mirb*ase.org/*).

BIOGENESIS OF MICRORNAS

The biogenesis of miRNAs consists of several steps from the nucleus to the cytoplasm. MiRNAs can be transcribed individually or in clusters. Based on their locations in the genome, miRNAs are classified as intergenic with their own promoter, or exonic and intronic. Their endonuclease synthesis is mediated through a Pol II transcription, resulting in a precursor molecule, called primary miRNA transcript (pri-miRNA) that shows a distinctive hairpin structure. Pri-miRNA is processed by a nuclear complex, comprising the DGCR8 protein (DiGeorge syndrome critical region 8) that recognizes the pri-miRNA and directs the cleavage by RNAse III endonuclease Drosha. The pri-miRNA processed by Drosha/GCR8 is converted into 60–100 nt hairpins, called precursor miRNAs (pre-RNAs), exported from the nucleus to the cytoplasm by Exportin 5/Ran-GTP action. Once there, pre-miRNA is cleaved by ribonuclease III enzyme Dicer/TRBP and turns out into a miRNA duplex of about 20 bp. Finally, the miRNA duplex merge with a protein named argonaute-2 (AGO2) and is incorporated into RNA-induced silencing complex (RISC), in which one strand is degraded, while the other constitutes the mature miRNA. Then, the miRNA-RISC complex recognizes specific targets, acting as regulators of gene expression and inducing gene silencing at posttranscriptional level.[77] Notably, miRNA biogenesis can follow an alternative noncanonical nuclear pathway through the generation of precursors derived from short hairpin introns, named "mirtrons."[78]

FINDINGS ON MIRNAS (INVOLVEMENT) IN ACM

Circulating miRNAs in human samples

The current interest in miRNAs as biomarkers is justified by their capacity to circulate extracellularly in biofluids, within membranous vesicles or associated with RNA-binding proteins and high-and low-density lipoprotein particles,[79] miRNAs integrity in the extracellular environment. During the last few years, several circulating miRNAs have been associated with ACM. Sommariva et al[80] performed a profile of circulating miRNAs in 2017 by evaluating the plasmatic expression of 377 miRNAs in ACM patients and healthy individuals, and then validating their results in 36 ACM, 21 IVT patients and 53 healthy controls. Only miR-320a showed statistically significant differences with 0.53- and 0.78-fold expression differences in ACM vs IVT patients and healthy cohorts, respectively. Briefly, these results suggested that miR-320a could be a novel potential biomarker for the disease, to distinguish between ACM and IVT. Of note, miR-320a was upregulated in human mesenchymal (skeletal or stromal) cells during adipogenic differentiation, thus suggesting mechanistic implications in ACM pathogenesis.[81] Similarly, Yamada et al[82] screened 84 cardiac-related miRNAs in a cohort of 62 patients (28 ACM, 11 borderline or possible ACM, 23 IVT) and 33 healthy patients. Four circulating miRNAs (miR-144-3p, miR-145-5p, miR-185-5p, and miR-494) showed higher expression levels in the ACM cohort compared to the other 3 groups. Interestingly, miR-494 was also a predictive factor of recurrent ventricular arrhythmia after ablation in ACM subjects.[82] According to these results, the screening of 754 miRNAs in the plasma of 21 ACM patients distributed based on their genotype or phenotype, versus 20 healthy individuals showed the overexpression of miR185-5p in the ACM plasma group.

One of the most solid studies on ACM and miRNAs screened miRNAs on right ventricular tissue and blood samples of 9 ACM patients and 4 controls. Afterward, a unique set of 6 circulating miRNAs was validated on 90 total blood samples from an independent cohort. This miRNA set showed high discriminatory diagnostic power in ACM patients when compared to controls, nonaffected family members of ACM probands carrying a desmosomal pathogenic variant, and other cardiomyopathy

groups such as hypertrophic cardiomyopathy, dilated cardiomyopathy and myocarditis. Concretely, miR-122-5p, miR-182-5p, and miR-183-5p were overexpressed in ACM compared to controls and the other cardiomyopathies, while miR-133a-3p, miR-133b, and miR-142-3p were downregulated in ACM compared to controls and other groups.[56] Only the ACM cohort fulfilled the miRNA set evolving as a potential biomarker for the clinical practice.

Cardiac miRNAs in human heart tissues

In 2007, the gap protein Connexin-43 (encoded by the *GJA1* gene) was involved with malignant arrhythmogenic events. Bioinformatics analysis demonstrated that miR-130 targets connexin-43, and it is up regulated in cardiomyopathies with heart failure.[83] In 2016, Zhang et al[84] performed the first expression profile of miRNAs on a cohort of 24 ACM patients who underwent cardiac transplants, compared to a group of 24 healthy controls. This study showed a set of 21 validated miRNAs as potential ACM miRNA signature, where 11 were significantly upregulated miRNAs (miR-21-3p, miR-21-5p, miR34a-5p, miR-212-3p, miR-216a, miR-584-3p, miR-1251, miR-3621, miR-3692-3p, miR-4286, miR-4301), and 10 were downregulated miRNAs (miR-135b, miR-138-5p, miR-193b-3p, miR-302b-3p, miR-302c-3p, miR-338-3p, miR-491-3p, miR-575, miR-4254, miR-4643) in an ACM heart, compared to a healthy control. Importantly, functional enrichment analysis established a correlation between the target genes of miR-21-5p and miR-135b and Wnt and Hippo signaling pathways, molecular mechanisms related to the ACM pathophysiology, suggesting a possible role in fibro-fatty substitution.[56] Although in the relevance of this study, a validation cohort was lacking and the arbitrary selection of miR21-5p and miR-135b over others with higher differential expression was not entirely justified (Table 3).

MiRNAs in ACM cellular models

In 2016, Gurha et al[85] performed the expression profile of miRNAs in 2 independent PKP2-deficient HL-1 cell (HL-1PKP2-shRNA) lines. Among the 59 miRNAs differentially expressed, miR-487b and 2 members of the miR-200 family (miR-200b and miR-429) were the most upregulated, whereas miR-184 and miR-881 were the most downregulated miRNAs. Consistent with the previous results, a study demonstrated by RT-qPCR that those 3 members of the miR-200 family, namely miR-200a-3p, miR-200b-3p, and miR429-3p, were upregulated in HL-1PKP2-shRNA cells.[86] However, only miR-200b inhibition fully restored the normal phenotype of HL-1PKP2-shRNA cells.[86] The expression of miR-184 was validated in the heart tissue of 2 ACM mouse models (Nkx2.5-Cre:Pkp2shRNA and Myh6:JupTr).[87] In 2019, Khudiakov et al[88] reported miRNA expression analysis in a conditioned medium collected from cardiomyocytes, differentiated from pluripotent stem cells of an ACM patient carrying a pathogenic alteration in the *PKP2* gene and a healthy donor. The expression of extracellular miRNAs miR-1 and miR-133a was increased in the conditioned medium obtained from ACM cells, suggesting myocardial damage. Also, higher levels of miR-21 and miR-29b-2, related to fibrosis were present in ACM supernatant, as well as miR-378a levels, a miRNA known to be expressed in adipocytes.[89] Of note, analysis of intracellular miRNAs showed no difference between the expression profile of cardiomyocytes from the ACM patient and healthy donor, highlighting that the differences appear upon secretion and not inside the cells.

MiRNAs in ACM animal models

In concordance to Gurha et al,[85] the expression of miR-184 was validated in the heart tissue of 2 ACM animal models (Nkx2.5-Cre:Pkp2shRNA and Myh6:JupTr).[87] In a transgenic murine heart model, miR-130a was overexpressed, leading to reduced connexin-43 levels over 90% in 10 weeks of life and induced ventricular arrhythmias. Lately, connexin-43 has been confirmed to be a direct target in fibroblast and cardiomyocytes assays.[90] Mazurek et al[92] reported that transgenic mice with specific overexpression of miR-130a in adult cardiomyocytes (αMHC-miR-130a) exhibited an ACM phenotype with increased fibrosis, lipid accumulation and cardiomyocyte death. In 2019, Calore et al[91] generated a novel murine model for ACM with cardiomyocyte-specific overexpression of human WT DSG2 protein (Tg-hWT) and p.Q558X_*DSG2* pathogenic variants (Tg-hQ). RNA-sequencing from myocardial samples showed the modulation of 24 miRNAs in Tg-hQ hearts compared to nontransgenic hearts, with 18 upregulated and 6 downregulated. This study identified a miRNA signature in murine ACM hearts, with miR-708-5p and miR-217-5p being the most upregulated and miR-499-5p the most downregulated miRNAs. All of them were predicted to be involved in the regulation of the Wnt/β-catenin pathway, adherence junction and gap junction, thus suggesting their potential involvement in the pathophysiology of ACM as well as their potential utility as future therapeutic targets for the disease.[91]

THE POTENTIAL ROLE OF MIRNAS IN THE ACM MOLECULAR PATHWAYS

MiRNAs are now recognized as critical regulators of diverse physiological and pathological processes; however, studies elucidating the potential biological relevance of miRNAs into the ACM pathophysiological mechanisms are still very few.

Desmosome and GAP junctions

Some of the desmosome and GAP junctions' proteins have also been predicted to be directly targeted miRNAs in the context of ACM pathogenesis. Concretely, a previous study showed that luciferase reporter assays identified DSC2 as a direct target of miR-130a[92](Fig 3) and immunofluorescent staining and western blot assays showed significant reduction of DSC2 expression compared to control. On the other hand, using this mouse model, the same group previously demonstrated that miR-130a overexpression in adult cardiomyocytes promoted atrial and ventricular arrhythmias and regulated Connexin-43 expression[90] (Fig 3).

Wnt/β-catenin pathway

Wnt/β-catenin pathway is one of the main molecular pathways underlying the ACM cellular phenotype. Suppression of the canonical Wnt/catenin-β1 signaling has been implicated in adipogenesis, fibrogenesis, and apoptosis.[17] Also, the activation of Hippo pathway has been associated with ACM pathogenesis, due to its effect on suppressing Wnt pathway, dysregulating cell proliferation/apoptosis and enhancing adipogenesis.[93] Several miRNAs have been suggested to be involved in the Wnt/β-Catenin pathway, being the miR-708-5p and miR-217-5p being the most upregulated and miR-499-5p the most downregulated miRNAs (Fig 2). All of them predicted to be involved in the regulation of Wnt/β-catenin pathway, adherence junction and gap junction.[91] On the other hand, *in silico* analysis showed that miR-185-5p was involved in the Wnt-Hippo pathway and cell junction process, leading to a structural functional impairment of the cardiomyocytes.[94]

TGFβ signaling

Several studies suggested that both canonical and noncanonical TGFβ signaling can have a role in inducing myocardial fibrosis in ACM. MiRNAs may play an important role in the regulation of these signaling pathways. In particular, under-expression of miR-26a and overexpression of miR-122 have been associated with angiotensin II-mediated fibrosis progression in the TGFβ-CTGF and LGR4 -β-catenin signaling pathways.[95,96] In these last 2 studies, Wei et al[96] reported that miR-26a is regulated by Nuclear Factor κB (NF-κB) signaling, a key regulator of inflammation and oxidative stress, and interacts directly with the 3′-

Table 3
The main research based on ACM and miRNAs

Patients/model	Source	Pathogenic alteration	Main findings	Reference
Screening of 377 miRNAs on ACM and HC subjects Validation on 36 ACM,[36] HC,[53] IVT[21] individuals	Plasma		miR-320a was overexpressed in ACM vs IVT. Not correlated with ACM severity	Sommariva et al[80]
Screening 84 cardiac-related miRNAs expression profiles in ACM[6] vs HC[6] Validation using RT-PCR on definitive ACM,[28] borderline ACM,[11] IVT,[23] HC[33]	Plasma Screening microarray Validation RT-PCR		Definite ACM patients had significantly higher expression of circulating miR-144-3p, 145-5p, 185-5p, and 494 miR-494 was overexpressed in definitive ACM with recurrent VA after catheter ablation	Yamada et al[82]
Screening 754 miRNAs in 4 ACM and 4 HC samples. Validation on ACM[37] and HC[30]	Plasma Taqman Array Human micro-RNA	*PKP2, DSP, DSG2*	miR-185-5p as a potential marker of ACM	Sacchetto et al[94]
Screening of miRNAs in 9 ACM and 4 HC using 84 miRNAs array Validation on the largest cohort ACM blood samples[90] and HC,[4] nonaffected family members and others cardiomyopathies	Heart tissue and plasma Small RNA sequence in tissue samples 84 miRNAs cardiac-related arrays for plasma by NGS analysis		miR-122-5p, miR-182-5p, and miR-183-5p were overexpressed in ACM	Bueno et al[56]
Screening 1078 miRNAs on 24 HTX and 24 HC	Myocardial tissue Using S-Poly(T) Plus method		11 upregulated: miR-21-3p, miR-21-5p, miR34a-5p, miR-212-3p, miR-216a, miR-584-3p, miR-1251, miR-3621, miR-3692-3p, miR-4286, miR-4301 10 downregulated: miR-135b, miR-138-5p, miR-193b-3p, miR-302b-3p, miR-302c-3p, miR-338-3p, miR-491-3p, miR-575, miR-4254, miR-4643. MiR-21-5p and miR-135b known to be involved as modulators of Wnt and Hippo pathways	Zhang et al[84]
Screening on 3 ACM patients and 3 HC. For validation, RT-qPCR measurements was performed of miR-520c-3p and miR-29b-3p in 8 AMC and 5 HC, that included those of the screening step	Cardiac stromal cells derived from endomyocardial biopsies of ACM patients Using TaqMan Low Density Arrays		miR-29b-3p in cardiac stromal cells contributes to ACM pathogenesis	Rainer et al[89]
Control and *PKP2*-knock down HL-1 (HL-1Pkp2-shRNA) cells were screened for 750 miRNAs	*PKP2*-deficient HL-1 cell (HL-1$^{PKP2\text{-}shRNA}$) lines Using low Taqman miRNAarray analyses		MiRNA-184, the most down regulated, was predominantly expressed in cardiac mesenchymal progenitor cells Knock down of miR-184 in HL-1 cells and MPCs induced and conversely, its over-expression attenuated adipogenesis	Gurha et al[85]
This study validated mmu-miR-200a-3p, mmu-miR-200b-3p, and mmu-miR429-3p upregulated in cells using RT-PCR (previous results Gurha et al)	HL-1 murine atrial cardiomyocytes (HL-1$^{Pkp2\text{-}shRNA}$) Using low Taqman miRNAarray analyses	*PKP2*	Mechanosensing in ACM is affected through miRNA-200 family targeting in *Itga1*	Puzzi et al[86]
Two pluripotent stem cells (iPSCs) lines from an ACM differentiated to cardiomyocytes and cultured in conditioned medium from ACM patient vs healthy donors	Cardiomyocytes derived from induced pluripotent cells of ACM patients Using TaqMan MicroRNA Reverse Transcription Kit	*PKP2*	No differences in intracellular miRNAs. Extracellular miRNAs, miR-1 and miR-133a over-expressed and miR-302 was downregulated miR-378a was overexpressed related to adipocytes	Khudiakov et al[88]
Arrhythmogenic phenotype in αMHC-miR130a mice	Transgenic mouse model capable of inducible overexpression of miR-130a in cardiomyocytes	*DSC2*	Overexpressed miR-130a repressed DSC2 genes leading to low concentrations of this protein and desmosomal dysfunction	Mazurek et al[92]
	Nkx2.5-Cre:Pkp2shRNA and Myh6:JupTr mice	*PKP2, JUP*	miR-184	Lombardi et al[87]
A murine model with cardiomyocyte-specific overexpression of a FLAG-tagged human *DSG2* was used to demonstrate 24 miRNAs dysregulated	Transgenic murine model RNA sequencing	*DSG2*	miR-217-5p and miR-708-5p were upregulated, whereas miR-499-5p was downregulated in Tg-hQ13 mice. In addition, miR-217-5p, miR-708-5p and miR-499-5p were predicted to be involved in the Wnt/β-catenin pathway	Calore et al[91]

Abbreviations: ACM, arrhythmogenic myocardiopathy; *DSG2*, desmoglein-2; *DSP*, desmoplakin; *HC*, healthy controls; *HTX*, heart transplant; *iPSCs*, pluripotent stem cells; *IVT*, idiopathic ventricular tachycardia; *NGS*, next-generation sequencing; *PKP2*, plakophilin-2; *RT-PCR*, reverse-transcription polymerase chain reaction.

UTR region of collagen I and CTGF mRNAs, conferring a regulatory feedback mechanism in cardiac fibrosis. In addition, Song et al[95] also found that overexpression of miR-122, increased loss of autophagy, cell migration, apoptosis and extracellular matrix deposition, proposing it as a biomarker and therapeutic approach in cardiovascular fibrosis and related diseases. Although the role of miR-122 in the ACM pathophysiological mechanisms is still to be fully understood, a previous study in ACM cases and controls heart samples concluded that miR-122 was one of the 6 signature miRNAs (miR-122-5p, miR-133a-3p, miR-133b, miR-142-3p, miR-182-5p, and miR-183-5p) presenting high discriminatory diagnostic power in ACM.[56]

Adipogenesis

Furthermore, miR-184 target genes associated with lipogenesis were upregulated with PKP2 knockdown, and in vitro models of aberrant

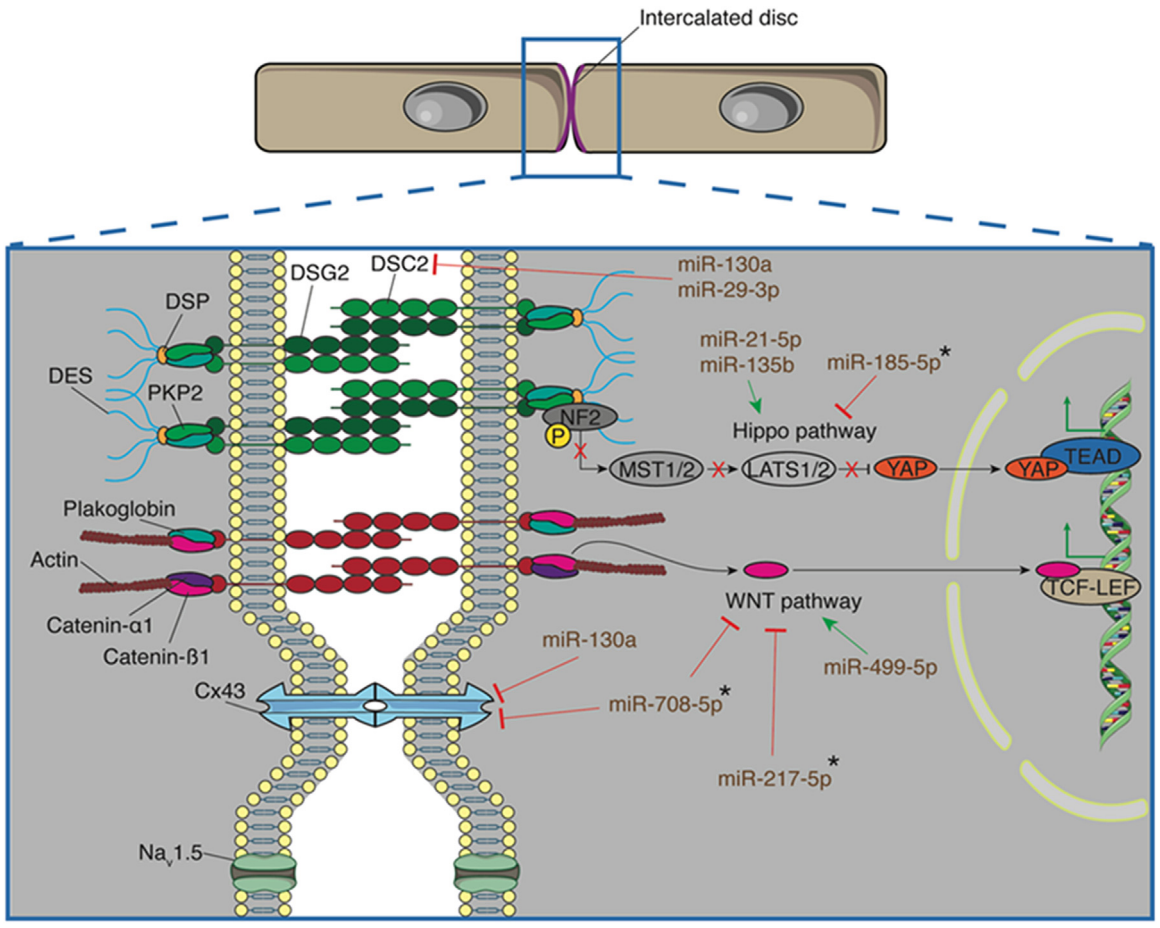

Fig 3. ACM and the 4 distinct structures in the intercalated disc involved in the pathology of this entity. Schematic flowchart of miRNAs orchestrating several mechanisms involved in ACM. Green and red means over and down expressed miRNAs. Cx43, connexion-43; DES, desmin; DSC2, Desmocollin-2; DSP, desmoplakin; DSG2, desmoglein-2; PKP2, plakophilin-2; *interaction no validated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

miRNA-184 expression illustrated its role as a molecular switch for adipogenic gene regulation.[85]

MAIN CHALLENGES OF MIRNA IN CLINICAL APPLICATION FOR ACM

MiRNAs are the most widely studied among all ncRNAs due to the fact that they are easily detectable in corporal fluids and tissues, cell-type specific, and they show distinct patterns and fluctuation based on physiological or pathological stress. Therefore, miRNAs can be studied using high-throughput miRNA sequencing or by arrays. The latter or real-time PCR are a fast, low-cost and reliable technique, very useful to screen miRNAs from various corporal fluids. On the other hand, high throughput miRNA sequencing provides a more complex and unbiased analysis, including global profiles of nonannotated ncRNAs[97] This analysis enriches the differential gene expression, alternative splicing, pathway findings and co-expression network.

Over the past decade, miRNAs have had massively increased attention as diagnostic biomarkers in cardiovascular diseases due to evidence of their potential role in pathogenesis. Multiple studies suggest that the circulating miRNA signature reflects the physiological or pathological status of a patient, stratifying their prognosis and tailoring personalized therapies. Besides, the analysis of miRNA patterns may help to understand the molecular mechanisms underlying the progression of the disease. Indeed, as we have discussed in this review, overexpression or suppression of miRNAs can contribute to the development of distinct cardiovascular disorders, including ACM. In this sense, miRNA mimics and anti-miRNA technology have triggered enthusiasm for miRNAs as potential drug targets. However, there are barriers to the clinical application of miRNAs. These include, for instance, the instability of miRNAs and the reproducibility of the results.

A handicap in daily practice is the absence of standardized methods, since the sample collection and storage, the total RIN value needed for an RNA analysis.[98] There are no accurate, consistent and robust methods for detecting and measuring circulating miRNA, highlighting the importance of standardizing guidelines as well as sample preparation and quantification methods.[99] In this regard, it has been shown that different methods of sample collection and storage result in a different quality of isolated RNA. Thus, the impact of RNA degradation and DNA contamination and, more importantly, low-input RNA analysis may influence the final results. Moreover, total RNA is frequently prepared from samples that were archived for various periods in frozen tissue banks. Unfortunately, the quality of samples stored in tissue banks is variable and RNA integrity is not always preserved, which will impact the reliability of the results of the analysis.[100] Besides, the differences between RNA isolation protocols affect the relative concentrations of miRNAs and limit the experimental reproducibility. On the other hand, the use of miRNAs to untangle their role in the pathophysiology as key regulators is limited by the experimental design, using animal or cellular models and their biological differences, and the inhomogeneity of miRNA depending on their origin. Human samples for any cardiovascular disease and ACM are usually restricted. However, even when they are accessible, several factors may influence the transcriptomic results, such as post-mortem interval of time, temperature, and handling protocols.

The lack of consensus regarding methodologies used for miRNA quantification is one of the main limiting factors. Three principal methods are used to measure the expression levels of miRNAs: real-time reverse transcription-PCR (qRT-PCR),[101,102] microarray hybridization,[103,104] and next-generation sequencing (NGS),[105] and each method has its limitations, thus affecting to the reproducibility of the results. Regarding circulating miRNAs, the lack of a universally invariant reference miRNA for normalization has become a serious challenge. Circulating miRNAs do not confirm their heart origin but show more specificity and sensitivity than other biomarkers. Nevertheless, consistent multicentric studies are required to achieve the size of an ACM sample that allows the establishment of a panel of miRNAs as biomarkers for ACM.[106] Intrinsic features linked to this entity, such as low prevalence, different penetrance, variety of genotypes and phenotypes, as well as segregation make it difficult to achieve this goal.

Finally, it is widely accepted the progressively increased use of miRNA as biomarkers in clinical practice. In this sense, the miRNA signature described by Bueno et al,[56] and the miR-185-5p reported by Sacchetto et al[94] have been proposed as promising miRNA for early diagnosis. However, all published studies using human samples have identified miRNA in patients showing ACM phenotypes but not necessarily with an early stage of the disease as well as, even before disease. Thus, the current knowledge in miRNA is probably not yet enough to propose miRNA biomarkers for early diagnosis of ACM.

CONCLUSION

In recent years, several data highlight the role of microRNAs in cellular processes of human physiology, despite the fact that a large part of mechanisms still remain to be clarified. Their potential role as biomarkers in different diseases has also been suggested in studies performed in different models, but no definite agreement among this wide variety of experimental studies exists to date, including in ACM. Variable expressivity and incomplete penetrance, hallmarks of this entity, even in families carrying a deleterious variant responsible for the disease, highlight the potential role of miRNAs as biomarkers.

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