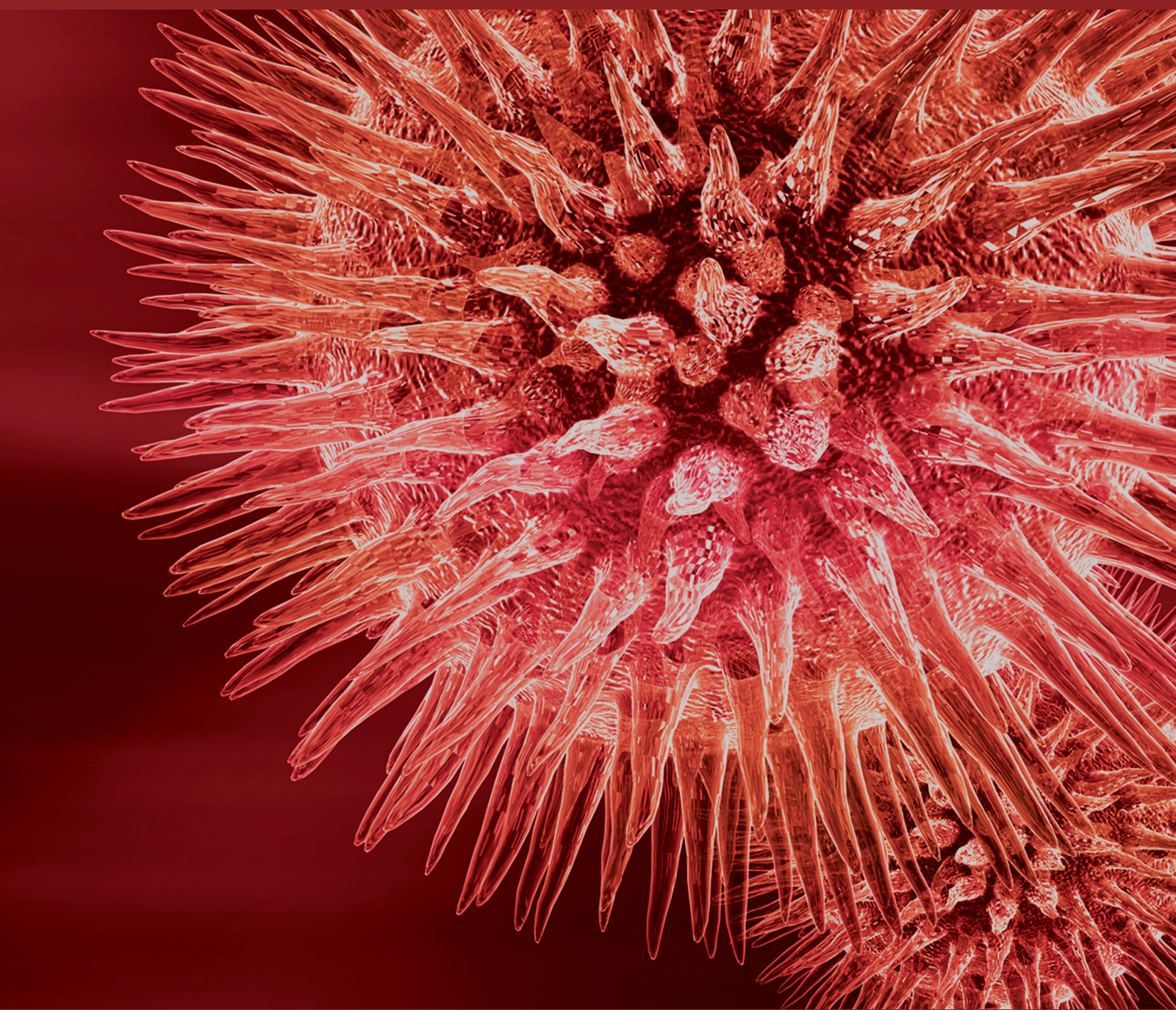
BioMed Research International

MicroRNA and Cardiovascular Disease 2016

Guest Editors: Ling-Qing Yuan, Vinicio A. de Jesus Perez, Xiao-Bo Liao, Magdalena Król, and Chi-Hsiao Yeh



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Editorial
MicroRNA and Cardiovascular Disease 2016

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A decade of research and development has shed light on the multifunctional characteristics and clinical importance of microRNA in cardiovascular diseases (CVDs). More and more evidence supports important roles of microRNAs in the pathogenesis, diagnosis, and potential treatment for various CVDs. This special issue includes several interesting studies and reviews on microRNA and CVD that aim at broadening the translational and clinical research frontiers in this exciting field.

Translational medicine aims at accelerating the implementation of basic science discoveries into clinical practice. The review by E. Cavarretta and G. Frati entitled "MicroRNAs in Coronary Heart Disease: Ready to Enter the Clinical Arena?" provides a systematic review on how miRNAs could serve as biomarkers on common CADs such as acute coronary syndromes and myocardial infarction. Their discussion includes consideration of the current technical challenges preventing their implementation in clinical practice, such as how to normalize miRNA values, how drugs could affect clinical measurements, and the range of values to be used in practice. While it is clear that miRNA-based therapy has great potential for CADs, there is still a long way to go before this becomes a reality.

Also, in this issue, X. Lin et al. have provided a review entitled "Function, Role, and Clinical Application of MicroRNAs in Vascular Aging," in which the role of microRNAs in the regulation of vascular aging is summarized, with a focus on how they impact the function of endothelial and vascular smooth muscle cells. Indeed, microRNAs are involved in modulating cell differentiation, proliferation, migration, senescence, apoptosis, and angiogenesis, all of which play critical roles in the pathogenesis of vascular aging. Furthermore, the potential application of microRNAs to clinical practice for the diagnosis and treatment of cardiovascular diseases is also discussed.

Epicardial adipose tissue, the metabolically active visceral fat around the heart, was proven to be associated with CADs and metabolic disorders. Y. Liu et al.'s study entitled "Role of miRNAs in Epicardial Adipose Tissue in CAD Patients with T2DM" used a microarray-based approach to identify microRNAs differentially expressed in epicardial adipose tissue in CAD patient with T2DM. Eleven microRNAs were selected for validation and their target genes were predicted using computational methods, which suggested that the insulin signaling pathway is potentially involved in the pathogenesis of CAD and metabolic disorders. Their study proposes that dysregulation of microRNAs in EAT might be associated with the pathogenesis of CAD and T2DM.

The study by A. Krajewska et al. entitled "Paroxysmal Atrial Fibrillation in the Course of Acute Pulmonary Embolism: Clinical Significance and Impact on Prognosis" analyzes the relationship and clinical implications between atrial fibrillation (AF) and acute pulmonary embolism (PE). They found that paroxysmal AF may be a sign of PE severity and may affect long-term prognosis. Their explanation is that sudden increase in right ventricular pressure results in a concomitant increase in right atrial pressure, leading to atrial tachyarrhythmia. Moreover, the author also found that PE

patients with AF have lower risk of deep vein thrombosis (DVT) compared with patients in normal sinus rhythm.

Y. Ding et al.'s review entitled "MicroRNAs and Cardiovascular Disease in Diabetes Mellitus" addresses the state of basic research and clinical studies on the contribution of microRNAs to CAD associated with diabetes mellitus. Abnormal expression of microRNAs induced by hyperglycemia is involved in endothelial cell injury, proliferation of VSMCs, platelet adhesion, and macrophage and lipid accumulation. The latter could trigger atherosclerosis and increase the morbidity and mortality of CAD in diabetes mellitus. Thus, microRNAs and their related gene targets could be potential biomarkers and therapeutic targets of CVD in patients with diabetes.

We hope these researches and reviews will bring new insights and spark ideas for future research and inspire readers who wish to pursue studies in the growing field of microRNA and CVD.

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Review Article

MicroRNAs and Cardiovascular Disease in Diabetes Mellitus

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Cardiovascular disease (CVD) is the major macrovascular complication of diabetes mellitus. Recently, although CVD morbidity and mortality have decreased as a result of comprehensive control of CVD risk factors, CVD remains the leading cause of death of patients with diabetes in many countries, indicating the potential underlying pathophysiological mechanisms. MicroRNAs are a class of noncoding, single-stranded RNA molecules that are involved in β-cell function, insulin secretion, insulin resistance, skeletal muscle, and adipose tissue and which play an important role in glucose homeostasis and the pathogenesis of diabetic complications. Here, we review recent progress in research on microRNAs in endothelial cell and vascular smooth muscle cell dysfunction, macrophage and platelet activation, lipid metabolism abnormality, and cardiomyocyte repolarization in diabetes mellitus. We also review the progress of microRNAs as potential biomarkers and therapeutic targets of CVD in patients with diabetes.

1. Introduction

Diabetes mellitus (DM) is a group of chronic metabolic diseases characterized by insulin deficiency and/or insulin resistance that leads to elevated blood glucose levels as well as abnormal fat and protein metabolism [1, 2]. Long-term hyperglycemia can result in both microvascular and macrovascular complications, of which cardiovascular disease (CVD) complications cause the most deaths in patients with diabetes. DM is also an independent risk factor for CVD, excluding other risk factors such as age, hypertension, and obesity [3, 4]. Most patients with CVD often have abnormal glucose metabolism, meaning diabetes and CVD are closely associated. Some studies have proved that high blood glucose levels stimulate the synthesis of advanced glycation end products, advanced oxidation protein products, and oxidation of low-density lipoprotein and that they are related to vascular injury in diabetes through several underlying processes that may be involved in the development and progression of atherosclerosis and could escalate the risk of CVD in patients with diabetes [5].

MicroRNAs are a class of noncoding, single-stranded RNA molecules containing 17–25 nucleotides that posttranscriptionally regulate their target genes by degradation or translational repression of the complementary messenger RNAs (mRNAs) [6]. In this manner, microRNAs modulate several physiological and pathological pathways in human disease, including diabetes, CVDs, cancer, and other diseases. Specifically, several microRNAs are involved in β-cell development and function, insulin secretion [7], and insulin resistance in the liver, skeletal muscle, and adipose tissue, which play an important role in glucose homeostasis and the pathogenesis of diabetes [8]. Altered microRNA expression also affects the progression of diabetic complications in the kidney, retina, and peripheral nerves. As each microRNA has the potential to regulate multiple genes in biological processes that include cell proliferation, differentiation, apoptosis, and development, it has been confirmed that the dysregulation of microRNAs affects many pathological pathways in diabetic complications [9].

In the last decade, it has been verified that numerous microRNAs play a pathophysiological role in CVD in

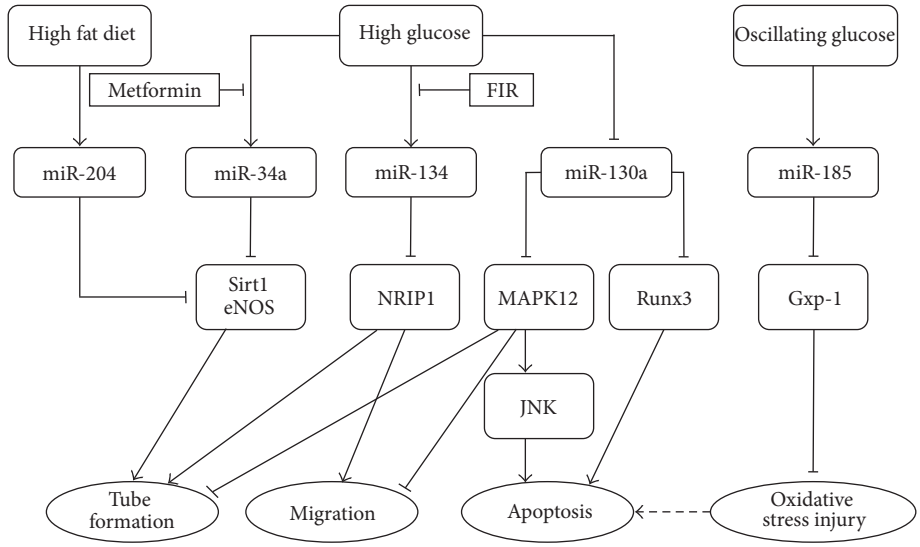

Figure 1: Schematic overview of the role of microRNAs in endothelial cells in hyperglycemia.

diabetes. Both in vivo and in vitro studies have shown that the abnormal expression of microRNAs induced by hyperglycemia causes endothelial cell, vascular smooth muscle cell (VSMC), platelet and macrophage dysfunction, and abnormal lipid metabolism. Clinical studies have indicated that some microRNAs could be diagnostic biomarkers of diabetes and diabetic macrovascular complications. In addition, microRNAs and the genes they regulate could be potential therapeutic targets for CVD in diabetes. In this review, we focus on recent studies in order to elaborate on the role of microRNAs in diabetes-associated CVD based on basic and clinical studies.

2. Basic Research on MicroRNAs in CVD in Diabetes

As the main pathological basis of CVD, atherosclerosis is a chronic inflammatory disease of the arteries caused by endothelial cell injury, proliferation of VSMC proliferation, platelet adhesion, and macrophage and lipid accumulation. It has been confirmed that the abnormal expression of microRNAs induced by hyperglycemia is involved in this abnormality. Furthermore, it appears that CVD is aggravated by arrhythmia caused by cardiomyocyte dysfunction, with the involvement of microRNA expression in high-glucose (HG) conditions.

2.1. MicroRNAs and Endothelial Dysfunction in Diabetes. Endothelial cells play an important role in the pathological progression of vascular complications resulting from diabetes. Several mechanisms of endothelial dysfunction in DM have been identified, including alteration in signaling related to endothelial nitric oxide synthase (eNOS) activation, increased oxidative stress, activation of the inflammatory processes, and impaired mitochondrial function. Arunachalam et al. [10] found that miR-34a was significantly increased in mouse microvascular endothelial cells in the presence of HG, accompanied by a significant decrease in SIRT1, which deacetylates and activates eNOS and results in impaired angiogenesis. Moreover, treatment with miR-34a inhibitor or metformin downregulated miR-34a expression and upregulated SIRT1 expression, indicating that the hyperglycemia-induced modulation of SIRT1 levels and posttranslational modification of eNOS took place through a miR-34a-dependent gene-regulatory mechanism (Figure 1). Vikram et al. [11] studied the role of miR-204 in endothelial dysfunction in high-fat diet-fed mice. Aortic miR-204 was upregulated, with impaired endothelium-dependent vasorelaxation and increased vascular inflammation. Further research found that miR-204 promotes endothelial dysfunction by targeting *Sirt1* (Figure 1).

Interestingly, a recent study found that miR-185 was upregulated in human umbilical vein endothelial cells (HUVECs) treated with oscillating glucose (OG), while it was unchanged when the cells were treated with HG as compared with cells treated with normal glucose [12]. OG also decreased glutathione peroxidase-1 (GPx-1), which plays a critical role in the enzymatic catabolism of reactive oxygen species (ROS). Cotransfection of HUVECs with the GPx-1 3′ untranslated region (3′ UTR) and miR-185 resulted in significant downregulation of luciferase light emission, indicating that GPx-1 might be a target of miR-185. Therefore, upregulation of miR-185 induced by OG targets GPx-1 to impair the antioxidant response and then caused endothelial cell injury (Figure 1).

Wang et al. [13] investigated the role of miR-134 in endothelial colony-forming cells (ECFCs) from patients with DM and from disease-free (DF) donors (dfECFCs). ECFCs from the patients expressed higher levels of miR-134 than that from the DF subjects and showed reduced cell migration and formation of microvasculature structure. dfECFCs treated with HG to mimic hyperglycemia or with HG combined with low growth factor (LGF) conditions (HG/LGF) to mimic the

poorest progression of patients with DM showed decreased cell mobility and tube formation ability, respectively. Subsequently, miR-134 overexpression in dfECFCs contributed to a 3.6-fold increase in miR-134 and reduced cell migration and tube formation ability. Similar findings were also found in HUVECs. Evidence from subsequent research showed that far-infrared radiation (FIR) treatment might suppress miR-134 expression and rescue ECFC function by at least 1.8- and 1.6-fold in terms of cell migration and tube formation ability, respectively. In addition, the *NRIP1* gene, which is involved in diabetes or angiogenesis, is believed to be a target of miR-134, where its expression is reduced in dfECFCs overexpressing miR-134 and is increased in ECFCs treated with FIR. Therefore, the increased miR-134 in HG conditions results in decreased NRIP1 levels, thereby impairing the angiogenic activities of ECFCs, which FIR treatment can reverse (Figure 1).

Xu et al. [14] examined microRNA expression in endothelial progenitor cells (EPCs), which play an important role in vascular repair. miR-130a was significantly downregulated in EPCs from the peripheral blood of patients with diabetes as compared with that of healthy individuals. miR-130a inhibition decreased EPC proliferation, migration, and colony formation but increased EPC apoptosis by targeting RUNX3. Downregulated miR-130a in EPC from patients with DM also dysregulated autophagy and autophagosome accumulation, an effect mediated by RUNX3, which could contribute to excessive autophagic cell death and impaired EPC function. Another study found that miR-130a inhibits the JNK pathway by targeting MAP3K12, contributing to its antiapoptotic effect and the maintenance of EPC function [15]. In diabetic EPCs, HG affects the expression of miR-130a, inducing sustained JNK activation and promoting EPC apoptosis and dysfunction. Consequently, the downregulation of miR-130a might underlie endothelial dysfunction in diabetes through activation of the JNK signaling pathway (Figure 1).

2.2. MicroRNAs and VSMC Dysfunction in Diabetes. The majority of cells in the tunica media of the arteries are composed of VSMCs, which are one of the most plastic cells in the human body. In hyperglycemic conditions, VSMCs change their phenotype from a contractile state to synthetic state, representing excessive proliferation, migration, and extracellular matrix secretion, which is believed to contribute to a series of pathological processes relevant to CVD [16]. Recent studies have proven that the modulatory mechanisms of HG-induced VSMC dysfunction are related to microRNA expression.

miR-504 levels were significantly upregulated in the aortic VSMCs of diabetic mice; miR-504 promotes VSMC proliferation and migration [17]. The coding gene for miR-504 is in the third intron of the *Fgf13* gene on the X chromosome. In addition, the study found that miR-504 targeted the *Grb10* and *Egr2* genes. In VSMCs, miR-504 overexpression downregulated the expression of *Grb10*, which enhanced ERK1/2 activation, thereby promoting the synthetic phenotype and increasing proinflammatory gene expression, proliferation, and migration. Modulated by miR-504

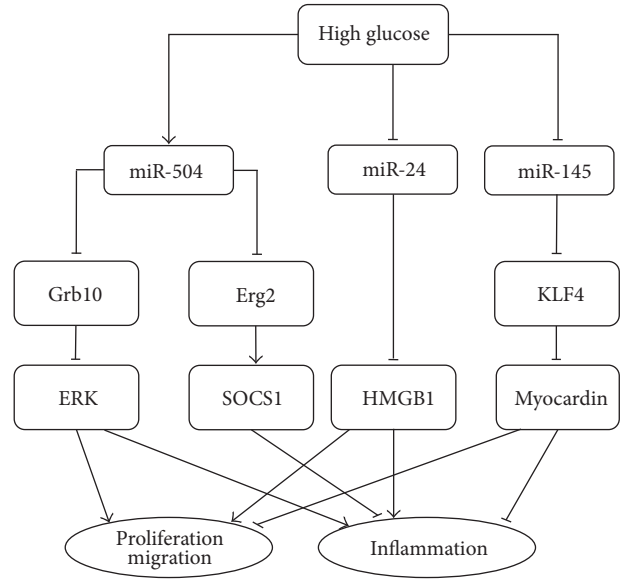

Figure 2: Schematic overview of the role of microRNAs in vascular smooth muscle cell in hyperglycemia or diabetes.

overexpression or *Grb10* gene silencing, *Egr2* downregulation reduced the anti-inflammatory *Socs1* and increased the expression of several proinflammatory genes, including *Il6*, *Ccl2*, and cyclooxygenase 2 *(Ptgs2)* (Figure 2). Therefore, the overexpression of miR-504 in diabetic mice might cause VSMC dysfunction by inhibiting the contractile genes and augmenting the inflammatory genes and proliferation and migration by downregulating *Grb10* and *Egr2* expression [17].

miR-24 is emerging as a regulator of VSMC pathology, targeting genes involved in HG-induced cell proliferation and migration. miR-24 expression was lost in HG-incubated VSMCs in vitro, which corresponded to an increase in HMGB1, a nuclear protein playing an important role in VSMC abnormal proliferation and migration [18] (Figure 2). Transfecting miR-24 into VSMCs markedly upregulated miR-24 expression and inhibited HG-induced VSMC proliferation and migration. As a downstream signaling molecule of HMGB1, NF-κB plays a key role in the inflammatory processes. miR-24 overexpression inhibited the HG-induced activation of NF-κB by suppressing NF-κB p65 translocation and NF-κB DNA-binding activity. Accordingly, miR-24 overexpression significantly decreased the HG-induced secretion of proinflammatory cytokines, including TNF-α and IL-6 [18].

Several studies have indicated the critical role of miR-145 in VSMC phenotype switching (Figure 2). Decreasing miR-145 increased the expression of its target gene *Klf4*. It increased *Klf4* and then decreased myocardin to induce VSMC proliferation and migration [19]. A recent study found that miR-145 expression differed according to glucose concentration and duration of glucose treatment [20]. miR-145 was decreased significantly by 12.5–75 mmol/L glucose, with 25 mmol/L glucose (HG) having the maximal effect. Subsequently, 25 mmol/L glucose decreased miR-145 expression

maximally at 0.5 h; after 4–6 h, miR-145 expression decreased gradually and was significantly lower than the control level [20]. These results indicate that sustained HG conditions decrease miR-145 expression in VSMCs. HG incubation increased angiotensin II (Ang II) secretion in VSMCs, which inhibited miR-145 expression under HG conditions. Furthermore, the addition of enalaprilat dihydrate and valsartan, an Ang II receptor antagonist, significantly upregulated miR-145 expression under HG stimulation [20].

2.3. MicroRNAs and Platelet Activation in Diabetes. Platelet activation plays a key role in the occurrence and development of CVD. Platelet activation, including adhesion, deformation, aggregation, and release, occurs when the vascular endothelium is injured or undergoes certain physiological and pathological stimuli, and this is an important initiating factor in thrombosis. The level of platelet miR-223 was attenuated in patients with diabetes and in mice, which affected platelet function [21]. Platelets from miR-223 knockout mice had greater aggregation and thrombi formation ability, along with longer clot retraction time as compared with platelets from the wild-type littermates [21]. In mice, miR-223 deletion resulted in the increased expression of proteins, such as β1 integrin, kindlin-3, and coagulation factor XIII-A (FXIII-A), which are elevated in individuals with diabetes as well. These proteins lead to a hyperreactive and hyperadhesive platelet phenotype. The altered miR-233 levels might have been related to the DM-induced activation of calpain. Calpain is a Ca^{2+}-activated cysteine protease that can lead to the proteolytic cleavage of Dicer, an RNAse III, to convert precursor microRNAs into mature microRNAs [22]. Treating diabetic mice with a calpain inhibitor significantly increased the level of platelet Dicer, as well as the expression of miR-233 and its target proteins, that is, kindlin-3 and FXIII-A. These results suggest that the loss of miR-233 enhances platelet reactivity in DM, which is modulated by the activation of calpain [21].

2.4. MicroRNAs and Macrophages in Diabetes. Macrophages are the main component of white blood cells in atherosclerotic plaques and are also the main cause of atherosclerotic plaque formation. Macrophages can be divided into M1 and M2 types. The former promotes inflammation and inhibits cell proliferation; the latter promotes cell proliferation and tissue repair [23]. The proportion of the two macrophage phenotypes affects the outcome of atherosclerosis [24]. Sun et al. [25] observed decreased expression of miR-181b in endothelial cells from the epididymal white adipose tissue (eWAT) of insulin-resistant mice and in HG-treated endothelial cells. Next, they demonstrated that the delivery of miR-181b could shift macrophage polarization toward the M2 anti-inflammatory phenotype and reduce macrophage accumulation in eWAT by targeting PHLPP2, which directly dephosphorylates and inactivates AKT at Ser473. However, miR-181b did not inhibit macrophage migratory and proliferative ability [25]. Therefore, decreased miR-181b in insulin-resistant mice increases vascular inflammation and accelerates atherosclerosis.

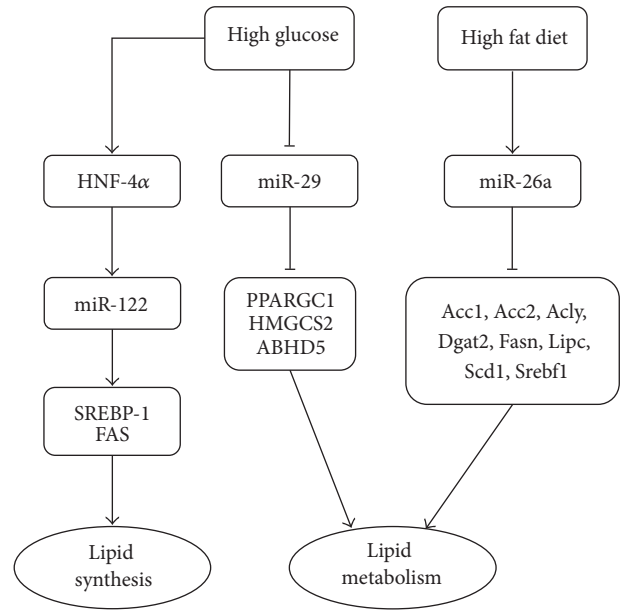

Figure 3: MicroRNAs involved in lipid metabolism.

2.5. MicroRNAs and Lipid Metabolism in Diabetes. Abnormality of lipid metabolism is another important risk factor for CVD in patients with DM. Several microRNAs are involved in lipid metabolism, mainly by regulating the expression of genes related to lipid synthesis, transport, and oxidation. The expression of hepatic miR-29 was upregulated in Zucker diabetic fatty *(fa/fa)* rats, and miR-29 was an inhibitor of FOXA2-mediated activation of key lipid metabolism genes, including *Ppargc1a*, *Hmgcs2*, and *Abhd5* [26] (Figure 3). Moreover, FOXA2 partly regulated hepatic miR-29 expression. FOXA2 activity was upregulated in insulin-resistant mice, which in turn elevated miR-29 levels [26]. This means that there is a miR-29 regulatory circuit in the liver and that miR-29 is an important regulatory factor in lipid metabolism.

Wei et al. [27] examined the role of miR-122 in regulating lipid metabolism. They found that miR-122 and hepatocyte nuclear factor-4α (HNF-4α) expression were increased in diabetic mice and in palmitate-treated HepG2 cells. HNF-4α is a nuclear receptor protein involved in liver development that modulates miR-122 levels in mouse liver [28]. HNF-4α and miR-122 expression in HepG2 cells upregulated the expression of SREBP-1 and FAS, which control cellular cholesterol homeostasis, and activated the genes that control fatty acid, cholesterol, and triglyceride synthesis [27] (Figure 3).

Another study demonstrated that miR-26a was downregulated in obese mouse models as compared with control animals [29]. The overexpression of miR-26a in mice fed a high-fat diet improved insulin sensitivity, decreased hepatic glucose production, and decreased fatty acid synthesis, thereby preventing obesity-induced metabolic complications. Conversely, silencing endogenous miR-26a in conventional diet-fed mice impaired insulin sensitivity, enhanced glucose production, and increased fatty acid synthesis. miR-26a targeted several key genes in lipid metabolism, including *Acc1*, *Acc2*, *Acly*, *Dgat2*, *Fasn*, *Lipc*, *Scd1*, and *Srebf1* (Figure 3).

2.6. MicroRNAs and Cardiomyocytes in Diabetes. Arrhythmia is characterized by abnormalities of cardiac origin, heart rate, and rhythm and impulse conduction. Dysfunction of the cell membrane ion channels is an important structural basis of arrhythmia. Li et al. [30] demonstrated that hyperglycemia led to electrophysiological change in cardiac progenitor cells (CPCs), which play an important role in the repair and regeneration of cardiovascular tissues. Furthermore, HG exposure induced augmented miR-1/133 expression in the CPCs. The overexpression of miR-1/133 suppressed KCNE1 and KCNQ1, which both encode the slow delayed rectifier potassium current (I_{Ks}), playing a key role in restoring the functional I_{Ks}, which is reduced by the inhibition of KCNE1 and KCNQ1 in diabetic conditions [30].

These studies indicate that microRNAs are differentially expressed and potentially have a pathogenic effect in diabetic CVD. Although many microRNAs have been identified, the mechanisms of their abnormal expression in DM and their target genes required further research and they should be fully characterized. These studies also showed that overexpression or inhibition of certain microRNAs could reverse its pathogenicity, which provides novel therapeutic approaches for alleviating diabetes-induced progression of cardiovascular complications.

3. Clinical Studies on MicroRNAs in CVD in Diabetes

In the previous sections, we reviewed the potential molecular pathology mechanisms of microRNAs in CVD in cytological and animal studies. These mechanisms indicate that microRNAs play a crucial role in the pathogenesis of atherosclerosis, which impedes the blood flow of coronary vasculature, followed by the occurrence of ischemic heart disease. Furthermore, epidemiological studies and clinical research on humans have demonstrated the involvement of microRNAs in CVD pathogenesis in diabetes.

3.1. MicroRNAs and Risk of Diabetes. A prospective population-based cohort including 80 patients with type 2 DM (T2DM) and 80 age- and sex-matched controls showed that, in patients with diabetes, miR-28-3p was overexpressed, and 12 other microRNAs were underexpressed [31]. Decreased circulating miR-126 was a significant predictor of DM. miR-15a, miR-29b, miR-126, and miR-223 were decreased in the subjects with DM [31]. In pancreatic β-cell, islets, enriched miR-375 was increased in subjects with T2DM and modulated β-cell function through several physiological mechanisms. miR-375 inhibits insulin secretion and transcription, maintains β-cell mass, proliferation, and regeneration, and promotes embryonic pancreas development [32]. Besides, it was found that microRNAs control the insulin signal transduction pathways in target tissues. Insulin resistance refers to the failure of target tissues, including the liver, skeletal muscle, and adipose tissues, to respond adequately to circulating insulin. Clinical studies have reported underexpressed miR-133 [33] and overexpressed miR-503 [34] in skeletal muscle, while increased miR-181a and decreased miR-17-5p, miR-132, and miR-134 have been observed in the omentum. In addition, miR-147 and miR-197 were increased in subcutaneous fat tissue while miR-27a, miR-30e, miR-155, miR-210, and miR-140 were decreased [35]. As the above findings suggest, microRNAs aid in the prognosis of diabetes and could be pharmacological targets in diabetes.

3.2. MicroRNAs and Risk of CVD. miR-126 is a human microRNA encoded in the intron of *Egfl7* that controls angiogenesis upon its various transcripts [36]. It is expressed more abundantly than other microRNAs in endothelial apoptotic bodies [37]. miR-126 mediates chemokine factor CXCL12 production, and shedding of miR-126 regulates vascular endothelial growth factor (VEGF) responsiveness and confers vascular protection [38]. The monocytes of patients with DM show impaired responsiveness to VEGF, which might be attributed to the reduced delivery of miR-126 to the monocytes. Recently, it was suggested that miR-126 could be a biomarker of coronary heart disease in patients with T2DM [38]. Compared with healthy controls, the expression levels of circulating miR-126 were decreased in the peripheral blood of patients with T2DM and in patients with T2DM with coronary artery disease (CAD). It has also been suggested that miR-126 correlates negatively with LDL in patients with CAD [39].

Polymorphisms in the corresponding sequence space in the form of single-nucleotide polymorphisms (SNPs) or mutations contribute significantly to phenotypic variation. miR-196a2 is a microRNA encoded by the MIR196A2 gene in humans. It belongs to the miR-196 precursor family, and miR-196a2 T/C polymorphism (rs11614913) is related to thrombosis and inflammation by regulating annexin A1 (ANXA1) in the circulation system [40]. Recently, a prospective case-controlled study of a Chinese population reported that the rs11614913 T $\rightarrow$ C variation in hsa-miR-196a2 is associated with poor prognosis of CAD [41]. Another study of a Chinese population found that a functional variant of miR-196a2 contributed to susceptibility to congenital heart disease [42]. Furthermore, common genetic polymorphisms in pre-microRNAs are associated with increased risk for dilated cardiomyopathy [43]. Regarding atrial fibrillation (AF), a study involving 123 participants showed that patients with AF with the TC + CC genotype had greater left atrial dimension than patients with the TT genotype, which supports the premise that the pre-miR-196a2 polymorphism is associated with AF and that the C allele is a risk factor for AF [44].

3.3. MicroRNAs and Risk Factors and Outcome of CVD. Genetic variants in microRNA genes or the 3′ UTR of microRNA target genes influence microRNA-mediated regulation of gene expression. In this manner, microRNAs influence the susceptibility and prognosis of human diseases. A study of 1004 hospitalized patients in China investigated the effect of microRNA-related polymorphisms on the prognosis of patients with angiographic CAD. The authors found that miR-4513 rs2168518 was associated with blood pressure, lipids, and blood glucose levels, and, as expected, risk for DM.

miR-499 rs3746444 and miR-423 rs6505162 were associated with blood pressure and high-density lipoprotein (HDL) levels. Event-free survival was apparently correlated with miR-4513 rs2168518 and miR-499 rs3746444. Furthermore, miR-4513 rs2168518 was associated with increased mortality in patients with CAD. Accordingly, miR-4513 rs2168518 and miR-499 rs3746444 might be biomarkers of the clinical prognosis of CAD [45]. In this case, the findings support the premise that microRNA-related polymorphisms influence clinical outcomes in CAD.

3.4. MicroRNAs and Pharmacotherapy of CVD. In the above sections, we reviewed studies clarifying how microRNAs mediate endothelial dysfunction in T2DM. It has been confirmed that this process is associated with the progression of atherosclerosis and neointimal proliferation in patients with T2DM after coronary stenting [46–48]. This emphasizes the importance of molecular targets in drug therapy for improving the endothelial dysfunction of such patients. A previous study observed early decreases in smooth muscle cell migration and proliferation when patients with T2DM were treated with pioglitazone [49]. A subsequent prospective study showed that pioglitazone significantly decreased neointimal hyperplasia (NIH), accompanied by increases in circulating miR-24. We can infer that decreased miR-24 might be associated with increased NIH in patients with T2DM [50].

In the clinic, there is interindividual heterogeneity of platelet response to clopidogrel. Clopidogrel is an irreversible P2Y12 receptor inhibitor. It mediates platelet glycoprotein IIb/IIIa (GPIIb/IIIa) inhibition by inhibiting adenosine diphosphate- (ADP-) induced P2Y12 activation of the downstream pathways and therefore influences vasodilator-stimulated phosphoprotein (VASP) phosphorylation. A recent study confirmed the existence of a microRNA pathway in anucleate platelets in humans and suggested that miR-223 regulates P2Y12 receptor expression [51]. It has been confirmed that decreased platelet miR-223 expression is associated with blunted platelet response to clopidogrel in patients with CHD [52]. In addition, a study of patients with troponin-negative non-ST elevation acute coronary syndrome showed that miR-223 levels correlated negatively with the platelet reactivity index (PRI). The results suggest that circulating miR-223 might be a novel biomarker for assessing the responsiveness to clopidogrel in such patients [53].

With solid evidence of the effects of microRNAs on the CVD pathophysiology increasing and the exciting development of potent microRNAs modulating technologies, microRNAs and the genes they regulate could be potential therapeutic targets for CVD in diabetes. Certainly, several challenges in the form of clinical complication remain. First, individual microRNAs should not be interpreted in isolation because microRNAs may work together, differentially, or in overlapping fashion. The challenge of establishing the role of these microRNAs in diabetic complications and identifying the regulatory mechanisms or pathways related to microRNAs expression in the pathogenesis of the various disorders remains. Second, more studies are needed to develop microRNA therapeutic methods with long half-life and tissue-specific to effectively deliver the microRNAs or their inhibitors to the cardiovascular system. In addition, the effectiveness and safety of long-term microRNAs overexpression or silencing in the clinic are unknown and require more intensive investigation.

Collectively, clinical studies on humans have demonstrated that microRNAs are related to the risk of CVD and could be potential biomarkers of CVD prognosis. Moreover, it has been suggested that microRNAs are correlated to the risk factors of CVD, such as blood pressure, blood glucose, and lipids. In terms of drug therapies, the varied responses correlated to microRNAs imply their potential clinical influence on individualized treatment.

4. Conclusions

In this article, we reviewed studies on how microRNAs are involved in CVDs through endothelial dysfunction, VSMC and cardiomyocyte dysfunction, platelet activation, macrophage phenotype, and lipid metabolism in diabetes. Despite the progress in lifestyle management and drug therapy, CVDs remain the most life-threatening complication of diabetes. This emphasizes the need for the integration of molecular research into the diagnosis and treatment of diabetic cardiovascular complications. Studies have explored the possible mechanisms in which microRNAs correlate with coronary heart disease, hypertrophic cardiomyopathy, and arrhythmias. Increasingly, studies have focused on how microRNAs modulate the function of endothelial cells, mast cells, and lipid metabolism. However, as studies among the diabetic population are limited, concrete mechanisms of how a particular microRNA affects different cell types and different cardiac diseases remain unclear. Furthermore, there is limited understanding of the cross-correlation of how different microRNAs act to date. The lack of clinical application also cannot be ignored. We anticipate further understanding of the pathophysiological mechanism in disease development and of the conversion of research findings to realistic predictions of cardiovascular risk and effective management.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Paroxysmal Atrial Fibrillation in the Course of Acute Pulmonary Embolism: Clinical Significance and Impact on Prognosis

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The relationship and clinical implications of atrial fibrillation (AF) in acute pulmonary embolism (PE) are poorly investigated. We aimed to analyze clinical characteristics and prognosis in PE patients with paroxysmal AF episode. *Methods*. From the 391 patients with PE 31 subjects with paroxysmal AF were selected. This group was compared with patients with PE and sinus rhythm (SR) and 32 patients with PE and permanent AF. *Results*. Paroxysmal AF patients were the oldest. Concomitant DVT varies between groups: paroxysmal AF 32.3%, SR 49.5%, and permanent AF 28.1% ($p = 0.02$). The stroke history frequency was 4.6% SR, 12.9% paroxysmal AF, and 21.9% permanent AF ($p < 0.001$). Paroxysmal AF comparing to permanent AF and SR individuals had higher estimated SPAP (56 versus 48 versus 47 mmHg, $p = 0.01$) and shorter ACT (58 versus 65 versus 70 ms, $p = 0.04$). Patients with AF were more often classified into high-risk group according to revised Geneva score and sPESI than SR patients. In-hospital mortality was lower in SR (5%) and paroxysmal AF (6.5%) compared to permanent AF group (25%) ($p < 0.001$). *Conclusions*. Patients with PE-associated paroxysmal AF constitute a separate population. More severe impairment of the parameters reflecting RV afterload may indicate relation between PE severity and paroxysmal AF episode. Paroxysmal AF has no impact on short-term mortality.

1. Introduction

Atrial fibrillation (AF) is the most common age-related, sustained cardiac arrhythmia. It accounts for 4% of cases of arrhythmia in the population older than 60 years and for 8% of cases in patients older than 80 years [1]. AF adversely affects the prognosis mainly because of thromboembolic complications such as stroke, development of heart failure, or progression of preexisting heart failure. The association between AF and acute PE is complex and not fully elucidated. The two conditions have some common risk factors, such as obesity, heart failure, myocardial infarction, and hypertension [2]. The risk of AF as well as pulmonary embolism (PE) increases with age. It has not been unequivocally explained whether the presence of AF in patients with PE affects their prognosis. So far, it has not been well established whether AF can lead to episodes of PE due to right-side intracardiac thrombi formation. Moreover, data concerning the prognostic significance of paroxysmal AF in patients with PE are sparse [3]. Paroxysmal AF may occur as a consequence of PE due to acute right ventricular (RV) systolic overload and subsequent right atrial dilation. Therefore, we decided to explore the hypothesis of whether paroxysmal AF could be a sign of PE severity and thus a marker of worse prognosis. We aimed to analyze the significance of paroxysmal AF that develops in the course of acute PE and to evaluate clinical characteristics of these patients, performance of the two prognostic scores for PE, and an impact of paroxysmal AF

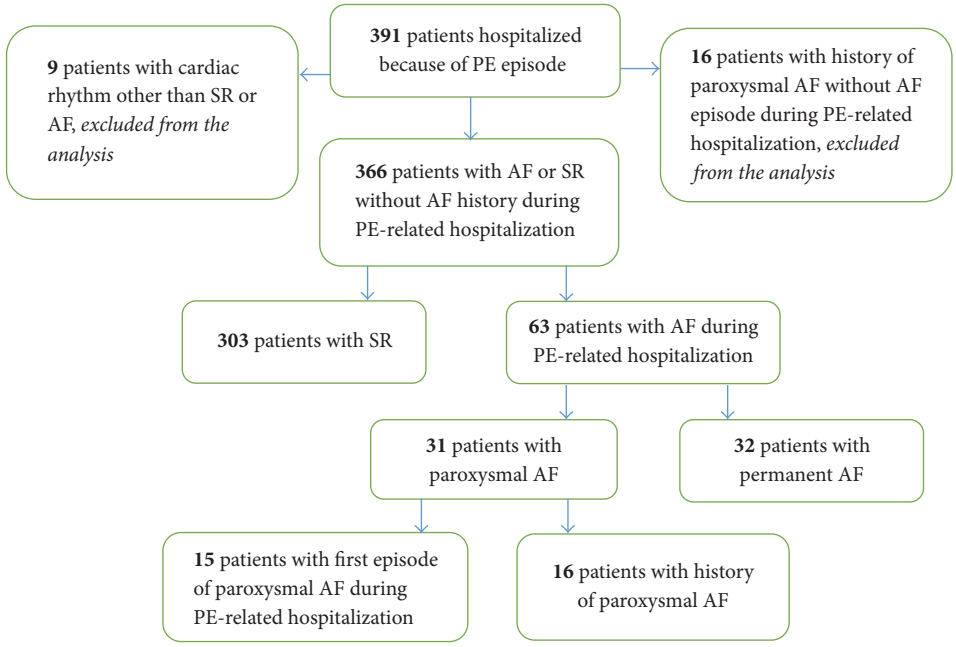

Figure 1: Study flow chart. The characteristics of the study population according to the heart rhythm. AF: atrial fibrillation; PE: pulmonary embolism; SR: sinus rhythm.

on short- and long-term all-cause mortality compared to patients in sinus rhythm (SR) and permanent AF.

2. Materials and Methods

The study cohort consisted of 391 consecutive patients with primary diagnosis of acute PE. Patients were aged 18 years or older and were hospitalized in the Department of Cardiology at University Hospital in Białystok, Poland, from January 1, 2004, to December 31, 2013. Their medical records were retrospectively analyzed. In 344 patients (88%), the diagnosis of PE was established by thoracic computed tomography angiography. In the remaining 47 patients (12%), it was confirmed on the basis of pulmonary ventilation-perfusion scintigraphy or echocardiography. As a standard procedure during index hospitalization, color duplex ultrasound of the lower extremity was performed to assess the presence of deep vein thrombosis (DVT). The study population was then divided into three groups (Figure 1):

(a) the sinus rhythm (SR) group included patients in SR throughout the hospital stay;

(b) the paroxysmal AF group included patients with one or more episodes of paroxysmal AF documented by electrocardiogram (ECG) at any time during index hospitalization. This group included patients in SR on admission who developed paroxysmal AF during hospital stay and patients with AF on admission with sustained SR during subsequent hospital stay;

(c) the permanent AF group included patients with the diagnosis of permanent AF confirmed by ECG.

There were no patients with valvular AF in the study cohort. In addition, patients with AF in history who did not develop AF during hospital stay were excluded.

On admission, demographic and clinical characteristics, including symptoms, hemodynamic profile, oxygen saturation, length of hospital stay, risk factors for PE, and comorbidities, were evaluated and compared between the groups. In addition, the probability of PE was evaluated retrospectively using the revised version of the Geneva score rule [4, 5], and the Simplified Pulmonary Embolism Severity Index (sPESI) was calculated retrospectively. The following laboratory parameters were also measured on admission: complete blood count, levels of troponin I, D-dimer, and estimated glomerular filtration rate (eGFR, using the Modification of Diet in Renal Disease formula).

Transthoracic echocardiography was performed within 24 hours of admission, and the following parameters were analyzed: left ventricular ejection fraction (LVEF) estimated by visual assessment, left atrial dimensions, presence of RV contractility disturbances, systolic pulmonary artery pressure determined using the simplified Bernoulli equation (SPAP = $4V\text{maxTR}^2$ + RAP, where VmaxTR is the maximal velocity of tricuspid regurgitant jet and RAP is the estimated right atrial pressure), pulmonary artery acceleration time, and presence of thrombi in the right heart cavities or in the pulmonary artery.

Finally, a standard 12-lead surface electrocardiogram was recorded to analyze the leading rhythm and the presence of tachycardia (>100 beats/min).

2.1. Study Outcomes. The primary outcome of the study was all-cause mortality. Data on all-cause in-hospital mortality were retrieved from medical records. The long-term outcome

Table 1: Characteristics and outcome of the patients with pulmonary embolism stratified for the presence of the sinus rhythm (SR), paroxysmal atrial fibrillation (paroxysmal AF), and permanent atrial fibrillation (permanent AF).

	SR (n = 303), Me (Q1–Q3) or %	Paroxysmal AF (n = 31), Me (Q1–Q3) or %	Permanent AF (n = 32), Me (Q1–Q3) or %	p value
Age, years	64 (49–76)[a,b]	78 (69–82)[b]	74 (67–79)[a]	<0.001
Males	44.2%	32.3%	43.8%	0.44
Obesity (BMI ≥ 30)	37.9%	41.7%	21.7%	0.27
Overweight (BMI 25–30)	25.0%	16.7%	26.1%	0.65
Current smoker	13,3%	7.7%	16.7%	0.62
Ex-smoker	23.8%	7.7%	25%	0.17
Length of hospital stay, days	9.0 (7–12)	10.0 (8–13)	9.0 (4–12)	0.13
Comorbidities				
Cardiovascular disease	12.9%	13.4%	15.6%	0.57
Arterial hypertension	54.8%	58.1%	56.3%	0.9
Diabetes	14.5%	16.1%	12.5%	0.92
Chronic obstructive pulmonary disease	5.3%	9.7%	3.1%	0.49
History of stroke	4.6%	12.9%	21.9%	<0.001
Mortality				
In-hospital mortality	5%	6.5%	25%	<0.001

Me (Q1–Q3) or %: data presented as a median and interquartile range or a percent of the group.
[a] p value < 0.01; [b] p value < 0.001.
BMI: body mass index (kg/m^2).

of the study cohort was retrieved from a national death registry database provided by the Polish Ministry of Home Affairs. A censored date of October 13, 2015, was determined to allow a minimum follow-up of 20 months for living patients (range, 20–178 months). In addition, we decided to analyze one-year survival.

The study protocol was approved by local ethics committee.

2.2. Statistical Analysis. In statistical analysis, categorical variables were compared using the chi-square test of independence. Normality of distribution was evaluated by the Kolmogorov-Smirnov test with the Lilliefors correction and the Shapiro-Wilk test. There was nonnormal distribution of continuous variables. The quantitative parameters were characterized by a median and quartiles and the nominal parameters using percentages. The nonparametric Kruskal-Wallis test with post hoc test was used to compare quantitative variables without normal distribution between the three groups.

Survival was estimated using the Kaplan-Meier method for each rhythm group. Differences between survival curves in the three groups were evaluated using the chi-square test.

For all tests, a p value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the Statistica 12.0 software (StatSoft, Inc., Tulsa, USA).

3. Results

The clinical characteristics, outcome, laboratory parameters, and risk score profiles of the study population stratified according to SR, paroxysmal AF, and permanent AF are shown in Tables 1–3. Of 391 patients with confirmed diagnosis of PE, we identified 63 individuals (16%) with AF. Thirty-one patients (7.9%) were classified as having paroxysmal AF, and 15 of them (48%) reported a history of AF. In 32 patients (8.2%), permanent AF was confirmed. Nine patients were excluded from the study as having cardiac rhythm other than sinus or AF (Figure 1).

Patients with paroxysmal AF were a median of 13 years older than patients in SR and a median of 4 years older than those with permanent AF (Table 1). There were no differences in sex distribution or in clinical symptoms on admission between the three groups (Tables 1 and 2).

The frequency of stroke in history significantly increased from 4.6% in patients with SR to 12.9% in those with paroxysmal AF and to 21.9% in those with permanent AF (Table 1).

Concomitant DVT during index hospitalization occurred significantly less often in patients with paroxysmal AF compared with the SR group. However, the proportion of patients with DVT was the lowest in patients with permanent AF (Table 2). The hemodynamic profile on admission revealed a higher prevalence of tachycardia (>100 beats/min) in both AF groups than in the SR group, with the highest proportion of patients with tachycardia in the group with paroxysmal AF (Table 2).

In the analysis of laboratory parameters, the only difference between the three groups was shown for eGFR: it was significantly lower in patients with paroxysmal as well as permanent AF as compared with those with SR (Table 3). Among echocardiographic parameters, patients with paroxysmal AF had the highest median value of estimated systolic pulmonary artery pressure and the shortest median pulmonary artery acceleration time (Table 3). On the other hand, patients with paroxysmal AF had significantly higher median values of left

Table 2: Comparison of the admission clinical parameters, Geneva risk score results, and sPESI score values in patients with sinus rhythm (SR), paroxysmal atrial fibrillation (paroxysmal AF), and permanent atrial fibrillation (permanent AF).

	SR (n = 303), Me (Q1–Q3) or %	Paroxysmal AF (n = 31), Me (Q1–Q3) or %	Permanent AF (n = 32), Me (Q1–Q3) or %	p value
PE symptoms				
Syncope	19.0%	26.7%	10%	0.25
Chest pain	30.0%	36.7%	30%	0.75
Dyspnea	86.6%	96.7%	79.3%	0.14
Hemoptysis	2.0%	0	6.3%	0.2
Cough	8.3%	6.7%	16.7%	0.28
PE associated with DVT	49.5%	32.3%	28.1%	0.02
Risk factors				
Immobilization	19.1%	19.4%	34.4%	0.13
Malignancy	17.8%	9.7%	18.8%	0.5
Pregnancy/delivery	3.3%	0	0	0.34
Recurrent PE	6.6%	0	3.1%	0.26
The revised Geneva risk score: clinical probability				0.04
Low	17.3%	16.1%	12.5%	
Intermediate	76.1%	64.5%	68.8%	
High	6.6%	19.4%	18.8%	
sPESI score ≥ 1	56.2%	86.2%	82.6%	<0.001
Hemodynamic profile on admission				
Heart rate, beats per minute	89.5 (78–103)[a]	99 (78–124)	101 (81–122)[a]	0.01
Tachycardia (>100 beats/minute)	32.0%	54.8%	50%	0.08
Systolic blood pressure, mmHg	130 (115–145)	125 (106–145)	126 (109–142)	0.6
Oxygen saturation, %	95 (92–97)	95 (90–97)	95 (90–96)	0.17

Me (Q1–Q3) or %: data presented as a median and interquartile range or a percent of the group.
[a] p value $p = 0.05$.
DVT: deep vein thrombosis; PE: pulmonary embolism.

Table 3: Comparison of the baseline laboratory and echocardiographic parameters in patients with sinus rhythm (SR), paroxysmal atrial fibrillation (paroxysmal AF), and permanent atrial fibrillation (permanent AF).

	SR (n = 303), Me (Q1–Q3) or %	Paroxysmal AF (n = 31), Me (Q1–Q3) or %	Permanent AF (n = 32), Me (Q1–Q3) or %	p value
Biochemical parameters				
eGFR, ml/min/1.73 m^2	76 (59–93)[c,d]	63 (51–83)[d]	53 (33–79)[c]	<0.001
Troponin I, ng/ml	0.066 (0.01–0.4)	0.11 (0.046–0.42)	0.036 (0.008–0.34)	0.3
D-dimer, ng/ml	5.6 (3–12.0)	10.6 (3.5–19.9)	11 (4.2–15.1)	0.3
Hemoglobin, g/dl	12.7 (11.4–14)	13.2 (11.4–14.7)	12.6 (10.7–15)	0.58
Echocardiography				
LVEF, %	60 (50–60)[c]	55 (50–60)[b]	48 (30–55)[b,c]	<0.001
LA, cm	3.7 (3.3–4.0)[a,c]	3.9 (3.6–4.4)[a,b]	4.4 (4.2–5.1)[b,c]	<0.001
SPAP, mmHg	47 (37–59)[b]	56 (47–70)[b]	48 (45–59)	0.01
ACT, ms	70 (54–95)[a]	58 (51–65)[a]	65 (55–80)	0.04
RV wall contractility disturbances	58.4%	77.4%	67.9%	0.09
Thrombus in RA/RV	5.2%	10%	14.3%	0.11

Me (Q1–Q3) or %: data presented as a median and interquartile range or a percent of the group
[a] p value < 0.05; [b] p value < 0.01; [c] p value < 0.001; [d] p value $p = 0.05$.
ACT: acceleration time; GFR: glomerular filtration rate; LA: left atrium; LVEF: left ventricular ejection fraction; SPAP: systolic pulmonary artery pressure; RV: right ventricle.

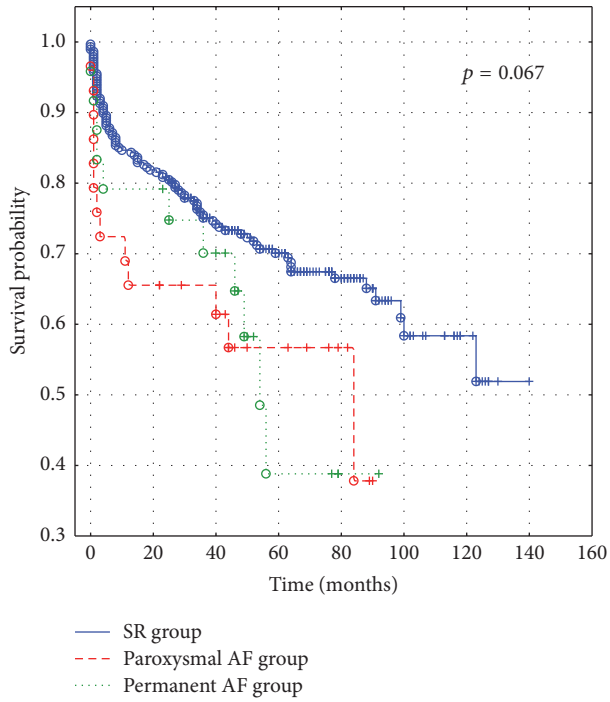

Figure 2: The Kaplan-Meyer curves in PE patients comparing survival between three groups: the sinus rhythm (SR) group, paroxysmal atrial fibrillation (paroxysmal AF) group, and permanent atrial fibrillation (permanent AF) group.

ventricular ejection fraction and smaller left atrial diameter compared with the permanent AF group (Table 3). Patients with AF also showed a tendency to have a higher rate of right heart thrombosis. Furthermore, this rate tended to be higher in patients with permanent than in those with paroxysmal AF, although the difference was not significant.

The retrospective analysis of the probability of PE, using the revised version of the Geneva score rule, showed significant differences between the three groups. Patients with both types of AF were more likely to be classified into the high-probability group than patients with SR. The high-probability group included more patients with paroxysmal than with permanent AF (Table 2). The same trend was shown for the sPESI. Eighty-six percent (86.2%) of patients with paroxysmal AF group had a sPESI of 1 or higher, compared with 82.6% of patients with permanent AF and 56.2% of those with SR (Table 2).

Regarding in-hospital mortality, it was significantly lower in patients with SR and paroxysmal AF (5% and 6.5%, resp.) compared to patients with permanent AF (25%) (Table 1). The results of the Kaplan-Meier survival analysis are presented in Figure 2. There was a trend towards worse survival in patients with paroxysmal AF in comparison with those with permanent AF and particularly those with SR, although the differences were not significant ($p = 0.067$).

4. Discussion

In this study, we found that, among patients with an acute episode of PE, those who develop paroxysmal AF on admission or during hospital stay have a different clinical presentation than those in SR or with permanent AF. We performed a detailed analysis of our baseline data, including the results of an echocardiographic examination of patients with paroxysmal AF and those with permanent AF. The data suggest that paroxysmal AF may be a sign of PE severity and may affect long-term prognosis. To date, no studies have been published that would focus specifically on paroxysmal AF in patients with PE. Regarding the analyzed variables, patients with paroxysmal AF are situated distinctly in between patients with SR and permanent AF. Interestingly, there were no significant differences between the three groups in terms of most comorbidities, risk factors, and symptoms of PE on admission, as well as the length of hospital stay.

The hypothesis that PE may provoke AF is grounded on a pathophysiological basis. Sudden RV systolic overload results in an increase of right atrial pressure, which in turn leads to atrial arrhythmias. In the present study, in an echocardiographic examination, patients with paroxysmal AF demonstrated the indirect signs of RV overload such as the shortest artery acceleration time and the highest estimated systolic pulmonary artery pressure compared with patients in SR or in those with permanent AF. Patients with paroxysmal AF also showed a trend towards the highest troponin I concentrations, although the differences with the other groups were not significant. Such results have never been demonstrated before.

In our study population the prevalence of AF was higher than in general population [1] and paroxysmal AF comprised nearly half of cases of AF. Depending on the inclusion criteria [3, 6, 7], the prevalence of AF in patients with PE was reported between 9% and 44%. Some investigators included both patients with AF on admission, as shown on an electrocardiogram, as well as those with a history of AF without AF at index hospitalization in a single AF group [6, 8]. Most authors did not differentiate AF into paroxysmal, persistent, and permanent [3, 6, 8–10]. There have been only a few studies investigating the relationship between PE and paroxysmal AF [6, 9–11]. In one study [5] the proportion of patients with paroxysmal AF was comparable. In another study paroxysmal AF was identified in 13% of participants [10]. The difference in the prevalence of paroxysmal AF was probably due to the design of the study, which investigated the quality of oral anticoagulation in a cohort of patients with a history of venous thromboembolism (VTE) at any time of their life. The problem of inefficient anticoagulation has been raised previously [12]. There were no data concerning the acuteness of a VTE episode and a temporal sequence of VTE and AF events.

AF may be not only a consequence of PE but also a risk factor for PE. It induces the prothrombotic state due to activation of the coagulation cascade and platelets [13]. Lack of atrial contraction results in blood stasis and possibility of thrombus formation in both atria, particularly in their appendages [14, 15]. Data concerning the association between PE, AF, right heart thrombus formation, and prognosis have been recently reported [5, 16]. Surprisingly, the authors did not find an association between AF and RHT. We observed a tendency to the higher prevalence of RHT both in patients

with paroxysmal AF and in those with permanent AF, but the difference did not reach significance.

Another indirect argument supporting the hypothesis about a causal relationship between AF and subsequent PE is the observation concerning lower frequency of concomitant DVT in patients with unprovoked PE [9–11]. In one study both paroxysmal AF and nonparoxysmal AF were associated with the increased risk of VTE (particularly PE), with the same statistical significance. In another study permanent AF was more common than paroxysmal AF in patients with isolated PE [10]. In our study in patients with SR, concomitant DVT was detected significantly more often than in patients with paroxysmal AF but the lowest frequency of concomitant DVT was discovered in patients with permanent AF.

Data on the effect of AF on outcome in patients with acute PE are rare and unequivocal. In some studies, negative impact on mortality was demonstrated [3, 6]. Some investigators did not find any association between AF in patients with PE and prognosis of these patients [10, 17]. Also, it is not known whether AF is an independent risk factor for mortality or whether it occurs as a consequence of PE severity or the presence of comorbidities. Furthermore, in the majority of studies concerning patients with PE, the effect of AF on survival was analyzed without differentiation of AF patterns.

There are several possible explanations why patients with paroxysmal AF in our study had worse echocardiographic parameters reflecting RV afterload and the highest proportion of an sPESI score of 1 or higher both indicating worse prognosis, but without impact on in-hospital mortality. One possibility is that both the signs of RV dysfunction on echocardiogram and sPESI help identify low-risk patients more accurately than high-risk patients [18–20]. Another possibility is that, in patients with SR and paroxysmal AF, there were two important prognostic makers whose median values were within the normal range: LVEF and eGFR [21].

Another important issue that emerged in our study relates to the usefulness of risk scores in PE patients with paroxysmal AF. We decided to verify the Geneva score rule, calculated retrospectively on admission, as well as the sPESI score. Their prognostic value in long-term follow-up was demonstrated among patients with confirmed PE [22, 23]. In our study the high-probability group included the highest percentage of patients with paroxysmal AF compared with the other groups. This probably resulted from older age and the prevalence of tachycardia (>100 beats/min) on admission in patients with paroxysmal AF. Despite the results of both scales, the in-hospital mortality rate of patients with paroxysmal AF was rather low, in contrast to patients with permanent AF in whom the rate was high.

The sPESI score was initially developed for 30-day risk assessment in patients with PE [24]. In our PE patients with paroxysmal AF, sPESI showed better prognostic value for long-term survival than for in-hospital outcome. Again it is possible that the presence of tachycardia typical for a paroxysmal episode of AF may constitute a confounding variable. This may mean that, in patients with PE complicated by a paroxysmal episode of AF, the current scales may overestimate short-term risk.

Our study has several strengths and limitations. The main limitation is the retrospective analysis of the patient's data. As a result, we have incomplete information concerning prior anticoagulation in patients with AF. On the other hand, the strengths of our study include a large number of patients from the same center, well-validated in-hospital data, and long-term follow-up.

5. Conclusions

Our study showed that individuals with paroxysmal AF constitute a separate population of patients than patients with PE and SR or those with permanent AF. Worse echocardiographic parameters reflecting RV afterload may indicate a causal association between the severity of PE and an episode of paroxysmal AF. This factor has significance but only for long-term prognosis. Further studies on a larger population of patients with PE are needed to determine the prognostic significance of AF types in patients with PE as well as the accuracy of PE risk scales in patients with different AF patterns.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Function, Role, and Clinical Application of MicroRNAs in Vascular Aging

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Vascular aging, a specific type of organic aging, is related to age-dependent changes in the vasculature, including atherosclerotic plaques, arterial stiffness, fibrosis, and increased intimal thickening. Vascular aging could influence the threshold, process, and severity of various cardiovascular diseases, thus making it one of the most important risk factors in the high mortality of cardiovascular diseases. As endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are the main cell biological basis of these pathology changes of the vasculature, the structure and function of ECs and VSMCs play a key role in vascular aging. MicroRNAs (miRNAs), small noncoding RNAs, have been shown to regulate the expression of multiple messenger RNAs (mRNAs) posttranscriptionally, contributing to many crucial aspects of cell biology. Recently, miRNAs with functions associated with aging or aging-related diseases have been studied. In this review, we will summarize the reported role of miRNAs in the process of vascular aging with special emphasis on EC and VSMC functions. In addition, the potential application of miRNAs to clinical practice for the diagnosis and treatment of cardiovascular diseases will also be discussed.

1. Introduction

Aging is a multifactorial process characterized by a progressive loss of physiological integrity and functionality, which increases mortality and susceptibility to diseases, including cardiovascular diseases, diabetes, osteoporosis, immunological diseases, various neurodegenerative diseases, and cancer [1–3]. Vascular aging is a specific type of organic aging playing a key role in the process of overall aging. Vascular aging is one of the most important risk factors in the high mortality of cardiovascular diseases and could influence the threshold, process, and severity of various cardiovascular diseases. Vascular aging is tightly linked to alterations in the biological functions and structural properties of the vascular wall, mainly including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). ECs, the inner layer of blood vessels, on the one hand, serve as a barrier between the blood stream and vessel and, on the other hand, regulate many aspects of vessel function, such as the control of vasodilation and vasoconstriction, inhibition of the adhesion of leukocytes and platelets to prevent blood coagulation, and suppression of vessel wall hypertrophy by inhibiting VSMC proliferation [4]. Furthermore, structural damage and dysfunction of ECs, such as senescence, apoptosis, proliferation, and inflammation, are closely associated with vascular aging. VSMCs, the main cells of the media vessel wall, can control blood flow by contracting or relaxing in response to external stimuli and play an important role in vascular pathologies. With increasing age, VSMCs are thought to undergo a phenotypic change from the quiescent, contractile phenotype to a synthetic phenotype. This synthetic phenotype is capable of migration into the intima and subsequent proliferation and extracellular matrix (ECM) synthesis, which in turn affects vascular function and disease outcome in the elderly [5]. Because vessels serve as transportation tools, they could supply nutrients, oxygen, and active substances and remove wastes or by-products and carbon dioxide produced in tissues [6]. Thus, vessels are critical to maintaining physiological homeostasis in vivo; as William Osler said, "a man is just as old as his arteries" [7]. Therefore, a better understanding of vascular

physiological and functional changes with aging is necessary and crucial to combat cardiovascular diseases in the future.

MicroRNAs (miRNAs), which are small noncoding RNAs, are approximately 18–25 nucleotides long. miRNA genes are mainly transcribed by RNA polymerase. They are initially transcribed as large precursors, called primary miRNAs (pri-miRNAs). Pri-miRNAs are then processed by the RNase-III enzymes Drosha and Dicer to generate mature miRNA products. Recently, studies have shown that miRNAs could cause target mRNA degradation at the posttranscriptional level and/or suppress the translation of mRNA into protein via interaction with the $3'$-untranslated region ($3'$UTR) of target mRNA by complementary base pairing [8]. So far, over 1000 miRNAs have been found in human cells. Each miRNA might target several genes and different miRNAs might target the same gene. As negative regulators of gene regulation, miRNAs contribute to many essential physiological and pathophysiological processes in humans, including differentiation, proliferation, apoptosis, migration, homeostasis, and various diseases [9, 10]. Therefore, it is not surprising that miRNAs are also involved in vascular aging and age-related diseases because of their multiple biological functions [11, 12].

2. miRNAs and Vascular Aging

Increasing evidence has shown that aging-associated physiological and functional disorders are associated with alterations in miRNAs, suggesting that miRNAs are novel cellular senescence regulators [59, 60]. Vascular aging is tightly linked to alterations in the biomechanical and structural properties of the vascular wall, including ECs and VSMCs dysfunction or apoptosis as well as increased arterial stiffness [11]. Until now, however, only the functions of a few miRNAs have been associated with cell dysfunction and/or vascular aging. In this section, we will discuss the role of miRNAs in the progression of vascular aging.

2.1. miRNAs and Endothelial Function. The vascular endothelium, a thin layer of ECs that lines the inner surface of blood vessels, is a critical interface between blood and all tissues. When the endothelium is exposed to various stimuli, such as hypoxia, proinflammatory cytokines, oxidative stress, hypertension, hyperglycemia, shear stress, aging, or injury, the function of ECs will be influenced, which is related to the proliferation, apoptosis, migration, senescence, angiogenesis, and inflammation of ECs [61]. Here, we will focus on individual miRNAs associated with endothelial functions (Table 1).

2.1.1. Influence of miRNAs on EC Apoptosis. EC apoptosis plays a vital role in the initiation and progression of atherosclerotic. In addition, EC apoptosis is responsible for plaque instability because EC death can predispose to arterial thrombosis, which could cause acute coronary occlusion and sudden death [62]. Accumulating evidence has indicated that miRNAs act as critical regulators to participate in EC apoptosis.

Several miRNAs are involved in the regulatory mechanisms of cellular apoptosis of ECs. Some are antiapoptotic miRNAs while others have proapoptotic effects. miR-126 was the most abundant miRNA in apoptotic bodies derived from ECs. It induced CXCL12 expression by targeting RGS16 and protected mice from atherosclerosis in a miR-126-dependent manner [63]. Recently, Chen et al. also demonstrated that miR-126 inhibits vascular ECs apoptosis through targeting PI3K/Akt signaling pathway [15]. miR-495 targets CCL2, significantly promoting human umbilical vein endothelial cell (HUVEC) proliferation and inhibiting apoptosis by affecting the expression of cleaved caspase-3 [19]. In addition, miR-19b plays a key role in the attenuation of TNF-α-induced EC apoptosis, and this function is closely linked to the Apaf1/caspase-7-dependent pathway [22]. Nevertheless, miR-132 promoted apoptosis of HUVEC induced by TNF-α and inhibited its proliferation, viability, and migration by inhibiting SIRT1 [23].

Oxidatively modified low density lipoprotein (Ox-LDL) is a major risk factor in the development of atherosclerosis. miR-365 and miR-US25-1 exerted a proapoptotic function in ox-LDL treated ECs by targeting the inhibition of Bcl-2 and BRCC 3 expression, respectively [26, 27]. Another miRNA, named miR-26a, was sufficient to reverse ox-LDL-induced apoptosis; the underlying mechanisms likely involved repression of TRPC6 and the associated downstream apoptotic pathway [14]. Furthermore, the let-7 family was found to be related to atherosclerosis and coronary artery diseases and is highly expressed in ECs. The inhibitory effects of let-7a and let-7b on ox-LDL induced EC apoptosis and dysfunction are partly obtained through the LOX-1/ROS/p38MAPK/NF-κB signaling pathway and the LOX-1/ROS/PKB/eNOS pathway [20]. Meanwhile, let-7g negatively regulated apoptosis in ECs by targeting caspase-3 expression [21]. In addition, miR-221/222 could partly alleviate apoptotic cell death mediated by ox-LDL through the suppression of Ets-1 and its downstream target, p21 [18].

Both intra- and extracellular conditions, such as shear stress, oxidative stress, hyperglycemia, and withdrawal, have a major effect on miRNA expression in EC functions, and the molecular mechanisms involved have been extensively studied [13, 16, 17, 24]. miR-21 targets PTEN and attenuates endothelial apoptosis by regulating Akt phosphorylation, eNOS phosphorylation, and NO production in human ECs [13]. Gα12 protects HUVEC from serum withdrawal-induced apoptosis by retaining miR-155 expression [17]. In diabetes patients, miR-130a inhibits the JNK pathway by targeting MAP3K12, contributing to its antiapoptotic effect and the maintenance of endothelial progenitor cell (EPC) function under high glucose conditions [16]. Other miRNAs have a proapoptotic effect on ECs. For example, miR-200c is upregulated by oxidative stress and induces EC apoptosis and senescence via ZEB1 inhibition [24]. Moreover, platelet-released miR-223 promotes advanced glycation end product- (AGE-) induced vascular EC apoptosis via targeting of IGF-1 [25].

2.1.2. Functions of miRNAs in EC Senescence. Senescence is associated with the cellular response to various environmental stressors and damage, which is defined as permanent cell cycle arrest. Senescent ECs are important in atherosclerosis and other age-related diseases [64]. An EC often undergoes

Table 1: miRNAs implicated in ECs functions.

ECs	miRNAs	Targets	Reference
	Cellular apoptosis		
Inhibit	miR-21	PTEN	[13]
	miR-26a	TRPC6	[14]
	miR-126	PI3K/Akt	[15]
	miR-130a	MAP3K12	[16]
	miR-155	Unknown	[17]
	miR-221/222	Ets-1/caspase-7	[18]
	miR-495	CCL2	[19]
	let-7a/b	LOX-1	[20]
	let-7g	Caspase-3	[21]
Promote	miR-19b	Apaf1	[22]
	miR-132	SIRT1	[23]
	miR-200c	ZEB1	[24]
	miR-223	IGF-1	[25]
	miR-365	Bcl-2	[26]
	miR-US25-1	BRCC 3	[27]
	Cellular senescence		
Promote	miR- 22	Vasohibin-1	[28]
	miR-34a	SIRT1	[29]
	miR-146a	SIRT1	[30]
	miR-200c	ZEB1	[24]
	miR-217	SIRT1	[31]
Inhibit	miR-92a	Unknown	[32]
	let-7g	SIRT1	[33]
	Cellular proliferation		
Promote	miR-29a	HBP1	[34]
	miR-126-5p	Dlk1	[35]
	miR-487b	THBS1	[36]
	miR-495	CCL2	[19]
Inhibit	miR-21	RhoB	[37]
	miR-24	Sp1	[38]
	miR-34a	SIRT1	[29]
	miR-92a	SIRT1	[39]
	miR-101	mTOR	[40]
	miR-125a	Bcl-2	[41]
	Cellular angiogenesis		
Proangiogenesis	miR-92a	PTEN	[42]
	miR-126	Spred-1	[43]
Antiangiogenesis	miR-15a	FGF2 and VEGF	[44]
	miR-20a	MKK3	[45]
	miR-21	RhoB	[46]
	miR-351	STAT3	[47]
	miR-214,	XBP1	[48]
	miR-223	β1 integrin	[49]
	miR-221/222	c-Kit	[50]
	miR-106	STAT3	[51]

Table 1: Continued.

ECs	miRNAs	Targets	Reference
	Cellular inflammation		
Promote	miR-21	PPARα	[52]
	miR-92a	SOCS5	[53]
Inhibit	miR-30-5p	Ang2	[54]
	miR-126	VCAM-1	[55]
	miR-155	Ang II type 1 receptor	[56]
	miR-181b	NF-kB	[57]
	miR-663	SLC7A5 and NAV2	[58]
	let-7g	TGF-β	[33]

EC, endothelial cell; PTEN, phosphatase and TENsin homologue; PI3K: phosphatidylinositol 3-kinase; TRPC6: transient receptor potential canonical 6; MAPK: mitogen-activated protein kinase; Ets-1: E26 transformation-specific 1; CCL2: C–C motif chemokine 2; LOX-1: lectin-like low-density lipoprotein receptor 1; Apaf-1: apoptotic protease-activating factor; SIRT1: silent information regulator 1; ZEB1: zinc finger E-box-binding homeobox 1; IGF-1: insulin-like growth factor-1; BRCC 3: BRCA1-BRCA2-containing complex; HBP1: HMG box-containing protein-1; Dlk1: delta-like 1 homologue; THBS1: thrombospondin 1; Sp1: specificity protein 1; mTOR: mammalian target of rapamycin; FGF2: fibroblast growth factor; VEGF: vascular endothelial growth factor; MKK3: mitogen-activated protein kinase kinases 3; STAT3: signal transducer and activator of transcription 3; XBP-1, a key unfolded protein response transcription factor; PPARα: peroxisome proliferator-activated receptor-α; SOCS5: suppressor of cytokine signaling 5; VCAM-1: vascular cell adhesion molecule 1; NF-kB: nuclear factor-kappa B; TGF-β: tumor growth factor-β.

both replicative and stress-induced presenescence. The function of miRNAs involved in the regulatory mechanisms of ECs senescence has been investigated. During replicative senescence of ECs, miR-22 could accelerate the process of aging by down regulating Vasohibin-1 [28]. However, miR-92a, a component of the miR-17-92 cluster, is highly expressed in young ECs. Rippe et al. reported that senescence of human ECs is associated with the reduced expression of miR-92a [32]. In the progress of stress-induced presenescence of ECs, miR-221 promotes senescence of human arterial ECs by inhibiting NO production and activating NF-κB signaling in human ECs [32]. Increased expression of miR-200c by ROS could induce the cellular senescence target zinc finger E-box-binding homeobox 1 (ZEB1) [24].

SIRT1 is a longevity gene that protects cells against oxidative and genotoxic stress. Recent studies have indicated that miR-34a is highly expressed in ECs. miR-34a expression is increased in senescent HUVECs and induces HUVEC senescence through the suppression of SIRT1 [29]. Two other miRNAs, miR-217 and miR-146a, promote senescence with a reduction of SIRT1 in ECs [30, 31]. On the contrary, let-7g has the effect of reducing EC senescence by increasing SIRT1 protein levels [33].

2.1.3. miRNAs and EC Proliferation. EC proliferation and viability are critical in the process of promoting endothelial healing and improving vascular function. Numerous lines of evidence support the involvement of miRNAs in EC proliferation. It has been reported that miR-495 significantly promoted HUVEC proliferation by directly targeting CCL2 [19]. Feng et al. demonstrated that miR-487b enhanced cell proliferation and migration in HUVECs through regulating THBS1 [36]. Apart from the influence on EC apoptosis, endothelial miR-126-5p could also promote the proliferation of ECs through suppression of the Notch1 inhibitor delta-like 1 homologue (Dlk1), thereby preventing the formation of atherosclerotic lesions [35]. Another highly expressed miRNA in endothelium is miR-29a, which was able to accelerate G1 to S cell cycle transition in HUVECs and enforce the expression of miR-29a in endothelium, remarkably promoting cell proliferation and angiogenesis via the targeting of HBP1 [34].

However, some miRNAs also exist that inhibit the proliferation of ECs. Both miR-34a and miR-92a are upregulated in ECs during aging, inhibiting cell proliferation and migration by targeting SIRT1 [29, 39]. Moreover, miR-21 can enhance the rapamycin-induced inhibition of endothelial proliferation by targeting RhoB [37]. In addition, miR-101 can induce cell cycle arrest at the G1/S transition and suppress mTOR expression and EC proliferation induced by laminar shear stress [40]. HUVEC proliferation is significantly inhibited by miR-125a and miR-24 via regulation of the expression of Bcl-2 and Sp1, respectively [38, 41].

2.1.4. Effects of miRNAs on Endothelial Angiogenesis. Angiogenesis is the process of new blood vessel and capillary network formation in the body, which is essential for recovery after cardiac and skeletal muscle injury or ischemia. Aged individuals, however, appear to have impaired physiological angiogenesis and are at higher risk of processes associated with pathological vessel formation, whereas ECs play a crucial role in the initiation of angiogenesis and the formation of early vascular structures [65]. A large number of miRNAs are responsible for angiogenesis and are expressed in ECs [43, 49–51, 66]. Wang et al. reported that miR-126, the endothelial specific miRNA, enhances the proangiogenic actions of VEGF and FGF and promotes blood vessel formation by repressing the expression of Spred-1, an intracellular inhibitor of angiogenic signaling [43]. Besides, members of the miRNA-17-92 cluster also exhibit a cell-intrinsic antiangiogenic function in ECs [42, 66, 67]. For example, pre-miR-92a treatment improves HUVEC viability and preserves angiogenic capacity under oxidative stress, at least partially through the downregulation of PTEN

expression [42]. Another study reported that miR-92a was identified as a negative regulator of angiogenesis by targeting the A5 integrin subunit (ITGA5) [67]. The contradictory results between the two studies might be attributed to the different functions of their different target proteins. Furthermore, miR-20a, another component of the miR-17–92 cluster, acts in a feedback loop to repress the expression of MKK3 and to negatively regulate p38 pathway-mediated VEGF-induced ECs migration and angiogenesis [45].

Other important miRNAs involved in angiogenesis regulation are the so-called antiangiogenic miRNAs, which include miR-221/222, miR-223, miR-206, miR-15a, miR-214, miR-21, miR-106b, miR-129-1, miR-133, miR-29c, miR-217, and miR-351. Poliseno et al. proved that miR-221/222 and miR-223 are antiangiogenic factors and that they affect the expression of the c-Kit receptor and β1 integrin in ECs, respectively [49, 50]. The signal transducer and activator of transcription 3 (STAT3) signaling pathway was regarded as a target for the prevention of atherosclerosis or other cardiovascular diseases. Previous studies showed that both miR-351 and miR-106b were upregulated in atherosclerotic mice and exerted an antiangiogenic effect in ECs by targeting STAT3 in vitro [47, 51]. Other miRNAs, such as miR-214, miR-21, and miR-15a, reduce angiogenesis of HUVEC by directly targeting XBP1, RhoB, and FGF2 and VEGF, respectively [44, 46, 48]. However, some miRNAs influence angiogenesis by affecting other functions of ECs. For example, miR-129-1 and miR-133 modulate angiogenesis by suppressing the proliferation rate, cell viability, and migration activity of HUVECs in vitro by targeting VEGFR2 and FGFR1, respectively [68]. Moreover, miR-29c plays a significant role in regulating angiogenic properties of HUVECs through the IGF-1/PI3K/AKT signaling pathway [69].

2.1.5. miRNAs Associated with Endothelial Inflammation. ECs, activated by shear stress, lipopolysaccharides, or cytokines, can modulate the expression of adhesion molecules and chemokines, leukocytes rolling over the endothelium and adhesion to vessels [70], which are stimulators of inflammation. Inflammation is associated with the development and progression of age-related conditions and they make individuals, especially the aged, more susceptible to cardiovascular diseases. Moreover, inflammatory mediators also play a fundamental role in the initiation, progression, and eventual rupture of atherosclerotic plaques and could therefore accelerate vascular aging [71]. Recent reports have shown that miRNAs can control vascular inflammation by controlling leukocyte activation and infiltration through the vascular wall [72]. Loyer et al. reported that miR-92a acts as a proinflammatory regulator in ECs by activating inflammatory cytokines and chemokines and promoting monocyte adhesion [53]. Zhou et al. showed that miR-21 suppresses the translation of peroxisome proliferator-activated receptor α (PPARα) mRNA, promoting endothelial inflammation by inducing the expression of vascular cell adhesion protein 1 (VCAM-1) and C–C motif chemokine 2 (CCL2) by increasing the activity of the transcription factor AP-1 [52].

Other important miRNAs associated with inflammation could inhibit endothelial inflammation. Harris et al. found that the inhibition of miR-126 increases proinflammatory TNF-α expression, which activates NF-κB and interferon regulatory factor 1 and finally induces the expression of VCAM-1 and the adhesion of leukocytes to ECs [55]. The systemic delivery of miR-181b also attenuates atherosclerosis by targeting NF-κB signaling in ECs [57]. miR-663, one of the oscillatory shear-sensitive miRNAs in HUVECs, is involved in oscillatory shear stress-induced cellular inflammation by regulating the potential targets of SLC7A5 and NAV2 [58]. miR-155 inhibits angiotensin II- (Ang II-) induced inflammation, migration, and apoptosis in HUVECs by targeting the Ang II type 1 receptor [56]. miR-30-5p acts in an anti-inflammatory manner in ECs induced by KLF2 and shear stress by impairing the expression of Ang2 and inflammatory cell-cell adhesion molecules [54]. Let-7g decreases EC inflammation and monocyte adhesion and increases angiogenesis via the TGF-β pathway [33].

Several important miRNAs regulate different kinds of EC functions among those that participate in the functional regulation of ECs. For example, miRNA-126 can inhibit apoptosis in ECs via the PI3K/AKT signaling pathway [15]. Meanwhile, it also plays a role in promoting angiogenesis and inflammation in ECs [43, 55]. In addition, miR-221/222 is also involved in the regulation of apoptosis, senescence, and angiogenesis in ECs [18, 50]. Upon summarizing numerous previous studies, it is not difficult to conclude that the SIRT1 gene, initially identified as a longevity gene, plays an important role in the regulation of ECs function. On the one hand, SIRT1 can be regarded as a regulatory target of multiple miRNAs, such as miR-34a, miR-221/222, miR-217, miR-132, and let-7g; on the other hand, it is involved in regulating multiple functions of ECs, such as senescence, apoptosis, and proliferation. Figure 1 shows the network of important miRNAs regulating the function of ECs.

2.2. miRNAs and VSMCs Function. VSMCs, the predominant cells in the tunica media of arteries, are highly specialized cells that represent the main contributor to vessel wall formation and vascular tension maintenance. The predominant phenotype of VSMCs is quiescent/contractile with nonmigratory and nonproliferative in periods of health. However, with the progress of aging and in response to various pathological stimuli, VSMCs deviate from their physiological state and switch to a proliferative, migratory, apoptotic, and differentiation phenotype, which is called phenotypic modulation or switching [73]. Recently, emerging evidence has revealed that miRNAs are involved in vascular disease through the regulation of VSMC migration, proliferation, differentiation, and apoptosis [74–77]. Next, we will summarize the current knowledge on the role of miRNAs in the regulation of VSMCs functions, including proliferation, migration, apoptosis, and differentiation (Figure 2).

2.2.1. miRNAs and VSMCs Apoptosis and Senescence. Apoptosis and senescence of VSMCs have been identified as important processes in a variety of human vascular diseases, such as atherosclerosis [78, 79]. ox-LDL plays an important role in atherogenesis. Studies have shown that hsa-let-7g can inhibit ox-LDL uptake and reduce apoptosis in SMCs

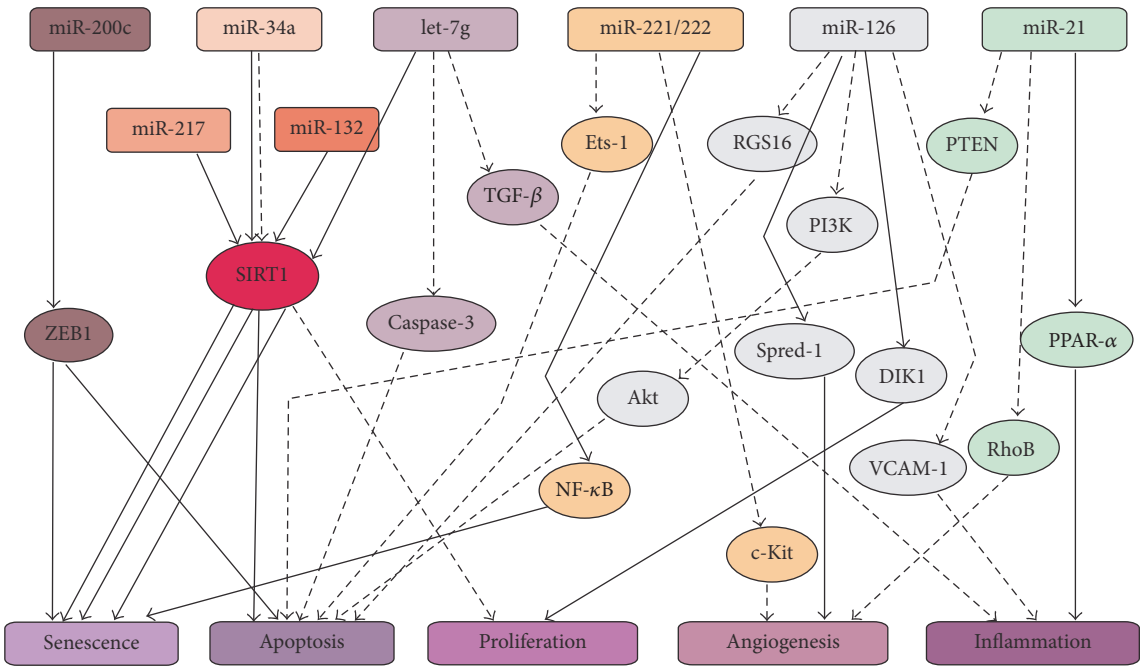

Figure 1: Network system of several important miRNAs regulating the function of ECs. The picture shows that SIRT1 is an important gene in the regulation of EC function. miR-34a, miR-217, miR-132, and let-7g are targets of SIRT1. Other miRNAs, such as miR-221/222, miR-126, and miR-21, participate in the function of ECs via targeting different genes. They can promote or inhibit the functions (senescence, apoptosis, proliferation, angiogenesis, and inflammation) of ECs. "—" indicates promotion effects; "---" denotes inhibition effects.

by the downregulation of cytochrome C and Smac/Diablo and upregulation of Bcl-2 expression [74, 80]. In addition, miR-34a, an aging-associated miRNA, can promote VSMCs senescence and inflammation through SIRT1 downregulation and senescence-associated secretory phenotype factor induction, respectively [76]. Moreover, miR-92a overexpression inhibits H_2O_2-induced VSMCs apoptosis and senescence by suppressing both mitogen-activated protein kinase 4 (MKK4) and JNK1 pathways [81]. Another miRNA, miRNA-146a was found to induce VSMC apoptosis via activation of the NF-κB signaling pathway [82].

2.2.2. miRNAs and VSMCs Proliferation and Migration. In the native vessel, VSMCs are maintained in a quiescent/contractile, nonmigratory and nonproliferative state. In response to vascular or mechanical injury, VSMCs switch to the dedifferentiated/synthetic phenotype and increase their ability to migrate to the intima space, proliferate, and produce the ECM, which contributes to the development of atherosclerosis. Therefore, the proliferation and migration of VSMCs are closely associated and together play a central role in the growth of atherosclerotic lesions. An increasing number of studies have demonstrated that miRNAs play an important role in the regulation of VSMC proliferation and migration [83–85].

(1) miRNAs That Promote the Proliferation and Migration of VSMCs. Some miRNAs have been found to promote the proliferation and migration of VSMCs. miR-21 is one of the most abundant miRNAs in the vascular wall following balloon injury; it can enhance VSMCs migration and proliferation caused by TSP-1 [86] and stimulate the proliferation and migration of VSMCs through the suppression of c-Ski [83]. c-Ski is a molecule that is expressed in VSMCs to suppress VSMC stimulation and intimal hyperplasia in a rat balloon injury model [87]. Therefore, in cultured human VSMCs, low expression of miR-21 significantly inhibits cell proliferation and migration by targeting different genes [88, 89]. miR-146a, a novel regulator of VSMC fate, promotes VSMCs proliferation and migration by targeting Krüppel-like factor 4 (KLF4) mRNA [90, 91]. Moreover, miR-146a and miR-21 were significantly upregulated in atherosclerotic plaques and cooperated to accelerate VSMC growth and cell cycle progression by targeting Notch2 and Jag1 [92]. Interestingly, miR-221/222, contrary to its effects of antiproliferation, antimigration, and proapoptosis in ECs, had the effects of proproliferation, promigration, and antiapoptosis in VSMCs. The different expression profiles of the target genes p27(Kip1), p57(Kip2), and c-kit between the two cell types might be related to the opposite effects [85, 93].

Diabetic VSMCs exhibit significantly increased rates of proliferation and migration, which is the most common pathological change in atherosclerosis. miR-138 promotes the proliferation and migration of VSMCs in db/db mice by suppressing the expression of SIRT1 [94], and miR-133a serves as a stimulatory factor for IGF-1R expression by prolonging the half-life of IGF-1R mRNA and promoting IGF-1-induced VSMC proliferation in murine atherosclerosis [95]. Therefore, identification of the miR-138 and miR-133a-IGF-1R pathways might provide insight into the design of

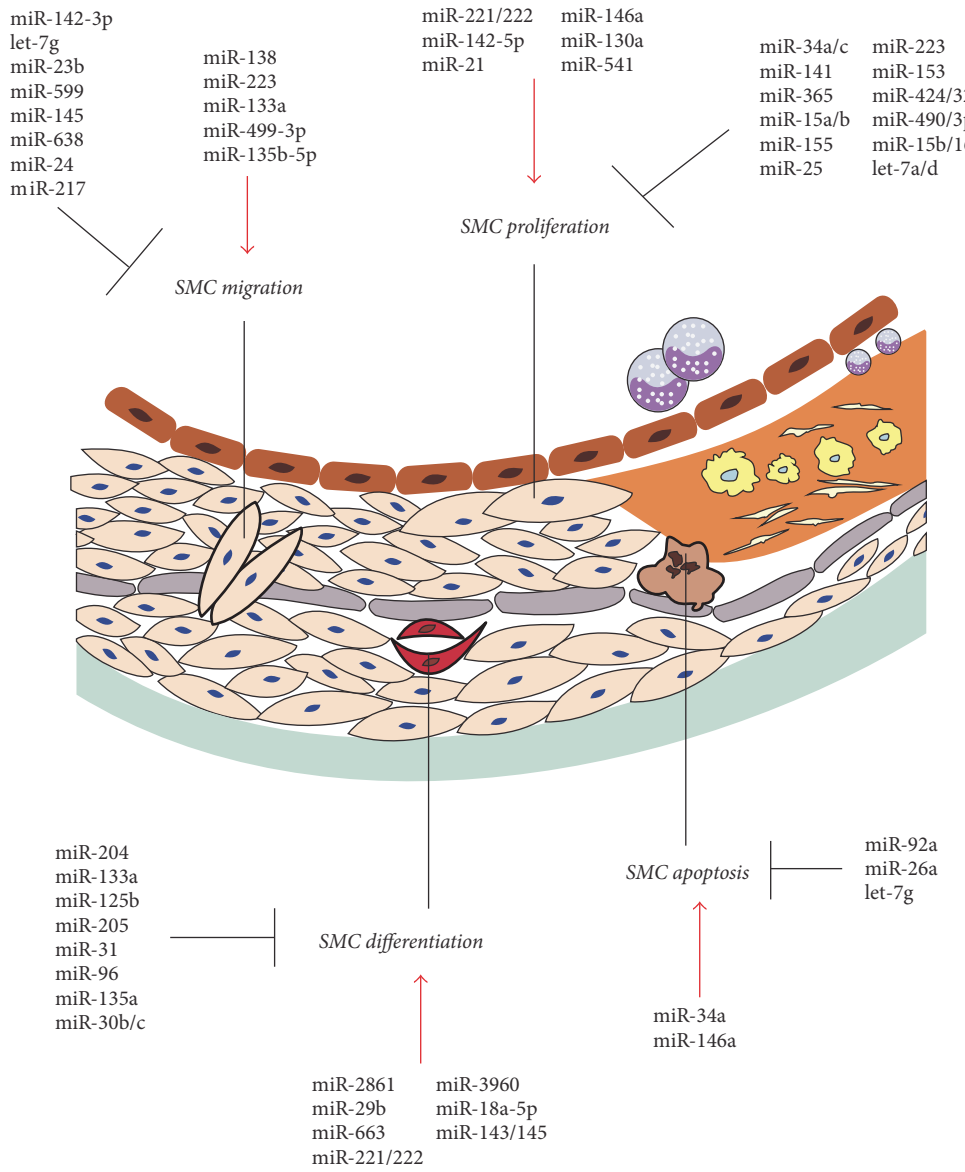

FIGURE 2: miRNAs that regulate phenotypic switching in VSMCs. The picture shows a variety of miRNAs that are involved in regulating the proliferation, migration, apoptosis, and differentiation of VSMCs. The red arrows indicate stimulatory effects, while the black arrows indicate inhibitory effects.

an efficient therapeutic approach to suppress atherosclerosis. In addition, other miRNAs, including miR-130a [96], miR-135b-5p and miR-499a-3p [97], miR-142-5p [98], miR-223 [99], miR-155 [100], and miR-541 [101], could promote VSMC proliferation and migration by regulating their own target genes.

(2) miRNAs That Inhibit the Proliferation and Migration of VSMCs. Other miRNAs have been reported to inhibit the proliferation and migration of VSMCs. By preventing VSMC proliferation, neointimal progression in atherosclerosis may be controlled. The let-7 family plays an important role in VSMC function. Let-7a decreased the proliferation of cultured VSMCs by reducing the expression of c-Myc and KRAS and could prevent intimal hyperplasia in an experimental vein graft model [102]. Overexpression of let-7d reduces VSMC growth by targeting KRAS [103]. Lower levels of let-7g have been observed both in subjects with hypercholesterolemia and in mice fed a high-fat diet. The transfection of let-7g into VSMCs has been shown to significantly inhibit VSMCs proliferation and migration induced by ox-LDL by targeting LOX-1 [104]. Moreover, both miR-141 and miR-490-3p could inhibit ox-LDL-induced VSMC proliferation through targeting of PAPP-α [105, 106].

Diabetes is another common age-related disease; VSMCs play a key role in the progress of diabetic atherosclerosis. miR-24 could inhibit high-glucose-induced VSMCs proliferation and migration by targeting high mobility group box-1 (HMGB1) [107]. Meanwhile, the G1/S transition activated by platelet-derived growth factor-BB (PDGF-BB) could be blocked by miR-365 [108] and miR-15b [109]. Additionally, miR-638 also mediated inhibitory effects on PDGF-induced cell proliferation and migration in human aortic SMCs by targeting the NOR1/cyclin D pathway [110]. Qian et al. reported that upregulating miR-542-3p in old VSMCs could inhibit VSMCs proliferation by directly targeting spleen tyrosine kinase. This downregulation of miR-542-3p may explain age-related neointimal hyperplasia in rats [111].

As mentioned in the miRNAs participate in the function of ECs, miR-34a and miR-34c inhibited VSMCs proliferation and migration through the modulation of Notch gene and stem cell factor (SCF) expression, respectively, which contributed to reducing neointimal hyperplasia [75, 112]. Specifically, overexpression of miR-223 and miR-153 inhibited stretch stress-enhanced VSMCs proliferation via activation of the insulin-like growth factor-1 receptor and PI3K-AKT signaling pathway [113]. Besides, miR-155 and miR-217 would inhibit angiotensin II and homocysteine-induced VSMC proliferation and migration [114, 115]. In contrast, miR-132 and miR-125b could block VSMC proliferation and neointimal hyperplasia in atherosclerosis [116, 117]. Other miRNAs that inhibit VSMCs proliferation and migration include miR-142-3p [118], miR-145 [119], miR-599 [120], miR-25 [121], miR-23b [122], miR-15b/16 [123], and miR-29b [124].

2.2.3. miRNAs and VSMCs Differentiation and Calcification. Vascular calcification is a highly prevalent phenomenon among the elder population and is identified frequently in patients with atherosclerosis, diabetes mellitus, and chronic kidney disease (CKD) [125–127]. One component of the vascular calcification process involves the reprogramming and transdifferentiation of VSMCs to osteoblast-like cells [128–131]. These osteoblast-like SMCs generate and release calcifying matrix vesicles that are another essential factor involved in vascular calcification [132–134]. As the process of vascular calcification is tightly regulated and involves the genetic reprogramming of VSMCs, it is not surprising that there is accumulating evidence to support an integral role for miRNAs in this process [135–138].

The transdifferentiation of VSMCs to osteoblast-like cells that from the bone matrix is a recognized contributor to vascular calcification. Our previous studies had demonstrated that miR-204 and miR-133a could reduce osteoblastic differentiation of VSMCs induced by β-glycerophosphoric acid (β-GP) via targeting runt-related transcription factor 2 (Runx2) [9, 10]. Wen et al. also identified that overexpression of miR-125b could inhibit β-GP-induced osteogenic marker expression and the calcification of VSMCs. Moreover, miR-125b targeted Ets1 and regulated its protein expression in VSMCs [138]. Furthermore, endogenous miR-205 inhibits the differentiation of HA-VASMCs into osteoblast-like cells by targeting Runx2 and Smad1, as evidenced by a decrease in ALP activity, osteocalcin secretion, and Runx2 expression [139], whereas miR-2861 and miR-3960 in VSMCs enhance β-GP-induced osteogenic transdifferentiation of VSMCs by targeting histone deacetylase 5 or Homeobox A2, respectively, resulting in increased Runx2 protein production [140]. The overexpression of miR-29b promoted Pi-induced VSMC calcification; thus, it plays an important role in the progression of vascular calcification via osteoblastic differentiation in VSMCs [136].

Many other miRNAs could be important biomarkers of diseases through modulation of the VSMCs phenotype. CREG and VSMCs differentiation marker gene expression levels were shown to be suppressed by miR-31 [141]. BMP signaling downregulates the transcription of miR-96, which in turn leads to upregulation of Tribbles-like protein 3 (Trb3), an essential positive regulator of the BMP signaling pathway, and promotes the contractile phenotype in VSMCs [142]. When overexpression of miR-663 and miR-18a-5p promotes VSMCs differentiation markers, SM α-actin and SM22α are involved in VSMCs differentiation by targeting JunB/myosin light chain 9 and syndecan-4 expression, respectively [143, 144]. mR145 acts to suppress TGFβ-dependent ECM accumulation and fibrosis, while promoting TGFβ-induced VSMCs differentiation [145]. At the same time, miR-145 and miR-143 cooperatively target a network of transcription factors, including KLF4, myocardin, and ELK-1 (ELK1, member of the ETS oncogene family), to promote differentiation and repress the proliferation of SMC [146], and VSMC differentiation marker genes such as SM-actin, calponin, and SM-MHC are upregulated by premiR-145 and miR-145 mediated phenotypic modulation of VSMCs through its target gene KLF5 and its downstream signaling molecule, myocardin [147].

Other miRNAs are involved in modulating the differentiation of VSMCs. For example, miR-135a acts as a potential osteogenic differentiation suppressor in senescent VSMCs by targeting both KLF4 and STAT3 [137]. Increased calcium deposition was observed in the combined treatment with mimics of miR-221 and miR-222 [135]. In VSMCs, miR-762, miR-714, and miR-712 were involved in calcification by disrupting Ca2t efflux proteins [148]. Additionally, BMP-2 downregulates miR-30b and miR-30c to increase Runx2 expression in VSMCs and promote mineralization and VSMCs calcification [149].

Several important miRNAs could regulate various functions of VSMCs. For instance, transfection of let-7g into VSMCs has been shown to significantly inhibit VSMCs proliferation and migration induced by ox-LDL by targeting LOX1 [104]. Moreover, let-7g can inhibit ox-LDL uptake and reduce apoptosis in SMCs via downregulation of cytochrome C [74]. In addition, miR-221/222 not only inhibits the differentiation of VSMCs but also promotes their proliferation and migration [85, 135]. In the process of regulating VSMCs function, the SIRT1 gene also has important effects on VSMCs, as it does on ECs. For example, miR-34a can promote VSMCs apoptosis by modulating the expression of SIRT1 [76], while miR-138 can promote the proliferation and migration of VSMCs by inhibiting the expression of SIRT1 [94]. Just like the SIRT1 gene, KLF4, a member of the family of evolutionarily conserved zinc finger-containing transcription factors, could be taken as a regulatory target of different miRNAs

to regulate the proliferation, migration and differentiation of VSMCs. For example, miR-146a could promote VSMC proliferation and migration by targeting KLF4 [91], whereas miR-15a acts as a direct transcriptional target of KLF4 that mediates the antiproliferative and antiangiogenic actions of VSMCs [150]. Meanwhile, miR-143 and miR-145 cooperatively target a network of KLF4 to promote differentiation and repress the proliferation of VSMCs [146].

3. How to Analyze the Role of miRNAs in Cells

The effects of miRNAs on the regulation of ECs and VSMCs are not a set of isolated processes; many miRNAs participate in modulating the function of both ECs and VSMCs including miR-221/222, miR-34a, miR-21, miR-217, miR-132, and the let-7 family. However, even the same miRNAs might have different effects on ECs and VSMCs. For example, miR-21 can enhance the rapamycin-induced inhibition of endothelial proliferation by targeting RhoB [37]. However, it can stimulate VSMCs proliferation and migration through suppression of c-Ski, and the low expression of miR-21 significantly inhibits cell proliferation and migration by targeting different genes [88, 89]. miRNAs, however, could also have a similar influence on ECs and VSMCs. For instance, let-7g negatively regulated apoptosis in the ECs by targeting caspase-3 expression [21]. Meanwhile, let-7g could inhibit SMC apoptosis by downregulating cytochrome C [74]. On the one hand, miR-221/222 could partly alleviate apoptotic cell death mediated by ox-LDL through the suppression of Ets-1 and its downstream target, p21 [18]. On the other hand, miR-221/222 also had antiapoptosis effects in VSMCs [85]. In addition, miR-34a expression is increased in senescent HUVECs and induces HUVEC senescence through the suppression of SIRT1 [29]; at the same time, it could inhibit cell proliferation and migration by targeting SIRT1 [39]. In VSMCs, miR-34a can also promote VSMCs senescence and inflammation through SIRT1 downregulation [76]. Furthermore, miR-34a inhibited VSMC proliferation and migration by modulating SCF expression [112]. The main explanation for the different roles of miRNA in ECs and VSMCs may be as follows: firstly, different cell types display their own unique characteristics and functions. Secondly, the particular structures and characteristics of different miRNAs play a key role in the functions of cells. Thirdly, the differing results may be related to the detailed conditions of the experiment. Finally, the target genes selected in the experiment may also influence miRNA functions. Different target genes have different biological properties; therefore, if miRNAs targeted the same genes in ECs and VSMCs, they will have similar effects. For example, miR-34a targets SIRT1 in both ECs and VSMCs; thus miR-34a has the same inhibitory effects on senescence and proliferation in the two cell types [29, 76, 112]. Nevertheless, different miRNAs with the same target genes may also produce different effects. For example, miR-146 can downregulate SIRT1 and promote ECs senescence [30], whereas let-7g, also with SIRT1 as the target gene, has inhibitory effects on ECs senescence [33]. Therefore, it is necessary to assess the specific environment and the target genes when analyzing the role of a miRNA.

4. Prospective Clinical Application of miRNAs as Diagnostic and Therapeutic Tool for Vascular Diseases

miRNAs have become one of the most important gene regulators involved in almost all types of cellular processes, including vascular cell differentiation, migration, proliferation, senescence, and apoptosis. miRNAs that are detected in serum or plasma are collectively called circulating miRNAs and the source of that might be vesicles (exosomes and microparticles), proteins, or lipoprotein complexes, which might fulfill biological functions outside the cell and act as potential biomarkers for cardiovascular diseases [151]. Although various tissues such as the heart, lung, liver, and kidney contribute to the circulating miRNA pool, most of the miRNAs are derived from blood cells [152].

It is generally considered that circulating miRNAs may provide a specific signature that reflects a given disease state; thus, measurement of circulating miRNAs can serve as a diagnostic tool in cardiovascular disease. For example, Li et al. investigated the relative expression of miRNAs in intima samples of peripheral artery disease patients and found that miR-21, miR-27b, miR-130a, miR-210, and let-7f were significantly upregulated, whereas miR-221 and miR-22 were decreased. In addition, miR-27b, miR-210, and miR-130a were increased in serum samples. Such miRNAs would be regarded as biomarkers for early atherosclerosis [153]. Moreover, those miRNAs (miR-204, miR-125b, miR-205, and so on) that inhibit vascular calcification are downregulated, while other miRNAs (miR-2861, miR-390, and miR-29b) that could enhance vascular calcification are upregulated. Therefore, measuring circulating miRNAs levels might be a method to diagnose vascular calcification. So far, there are three major methods that could be applied for circulating miRNA identification and quantification. One is microarray technology, which has been utilized to provide a comprehensive miRNA expression profile. The other is real-time quantitative PCR (qRT-PCR), which is a simple tool that can efficiently determine the amount of a gene transcript in a given sample. The third one is next-generation sequencing (NGS), which provides us with an opportunity to examine all miRNA variants simultaneously, thereby helping in the identification of novel, disease-related miRNAs [154]. However, there are also some problems using these technologies to measure the circulating miRNAs. Firstly, the simplicity of this methodology can itself be problematic [155]. For example, there is no consensus as to whether plasma or serum is a more reliable substrate for measuring circulating miRNAs. Secondly, hemolysis during sample preparation, or even due to physiological processes, can also affect the levels of circulating miRNAs [156]. Moreover, antiplatelet treatment may affect circulating miRNAs in plasma and serum samples and may act as a confounding factor in case-control studies relating plasma miRNAs to cardiovascular disease [157]. Finally, different tissues could express the same miRNAs, which are all transmitted to the blood; thus, the measurement of circulating miRNAs lacks specificity. Therefore, there is a long way to go to increase the diagnostic accuracy of circulating miRNAs to diagnose cardiovascular diseases.

Specific miRNA expression can be modulated by genetic approaches including overexpression or silencing of the prospective miRNA [158]. Thus, delivery of miRNA mimics into the proper tissue can provide a therapeutic benefit by enhancing the levels of specific miRNAs whose expression is downregulated in the disease state. Chen et al. demonstrated that overexpression of miR-126 inhibits vascular ECs apoptosis through targeting of PI3K/Akt signaling [15]. Consistent with this study, adenovirus-mediated restoration of miR-145 into rat balloon-injured carotid arteries in vivo significantly inhibited neointimal lesion formation [147]. Nevertheless, for specific miRNAs that are upregulated during disease, silencing of specific miRNAs would be beneficial. Currently, modified oligonucleotides can be designed to complement either the mature miRNA or its precursors leading to the inhibition of specific miRNA [159]. Liu et al. have applied modified antisense oligonucleotides to successfully knock down miR-221 and miR-222 in cultured VSMCs and significantly inhibit cell proliferation and neointimal growth in rat balloon-injured carotid arteries [85]. However, because miRNAs are endogenous, restoration of aberrantly expressed miRNAs, both upregulated and downregulated, to physiological levels cannot be achieved without some unexpected side effects. For instance, the inhibition of a specific miRNA may be beneficial concerning atherosclerosis progression but may adversely affect other organ systems causing immunosuppression, liver damage, or even oncogenesis.

It is well known that miRNAs have an inhibitory effect in their targets mRNA transcription and consequently, on gene expression. In other words, the inhibition of miRNAs induces gene expression while the addition or enhancement of miRNAs has the opposite effect. Hence, the greatest challenge here lies in the ability to predict the exact effects of miRNA modulation in the human body. However, one miRNA can have multiple targets; for example, miR-21 can enhance the rapamycin-induced inhibition of endothelial proliferation by targeting RhoB [37]. Meanwhile, miR-21 significantly inhibited VSMC proliferation and migration by targeting tropomyosin and AP-1 [88, 89]. One gene can also be regulated by several miRNAs. For instance, miR-217 and miR-146a promote senescence with a reduction of SIRT1 in ECs [30, 31], whereas let-7g has an effect on reducing ECs senescence by increasing the SIRT1 protein [33]. Keeping this in mind, miRNA-based therapy may have both advantages and disadvantages. miRNAs that have only a single target gene should be easy to suppress using anti-miRNA technology, which represents an advantage. However, the suppression of miRNAs that have multiple target genes will affect several genes and might induce some unexpected side effects, which could be a disadvantage [160]. Therefore, although targeting miRNAs represents promising therapeutic strategies, careful monitoring and studying of these interactions is essential in order to guarantee a safe application in humans.

5. Conclusion

Aging and its associated diseases remain a huge burden especially within the next decades; research efforts are increasing to identify the underlying molecular mechanisms and especially innovative treatment approaches to diseases closely associated with aging. To date, accumulating evidence has revealed that miRNAs are becoming one of the most fascinating areas of biology and play a crucial role in regulating aging processes in animal models and humans. The relative role of different miRNAs in vascular biology as direct or indirect posttranscriptional regulators of genes implicated in structural remodeling, inflammation, angiogenesis, atherosclerosis, in-stent restenosis, and thrombosis indicates that miRNAs may serve as promising drug targets or potential biomarkers in the prevention and management of vascular disorders. In this review, we have summarized the roles of miRNAs in the regulation of vascular aging, especially with respect to EC and VSMC functions, including differentiation, proliferation, migration, senescence, and apoptosis, all of which play critical roles in the pathogenesis of vascular aging. With rigorous fundamental and clinical studies, a clearer understanding of miRNAs as biomarkers and targets for cardiovascular disease will provide new insight into vascular aging and aging-related diseases.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

MicroRNAs in Coronary Heart Disease: Ready to Enter the Clinical Arena?

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Coronary artery disease (CAD) and its complication remain the leading cause of mortality in industrialized countries despite great advances in terms of diagnosis, prognosis, and treatment options. MicroRNAs (miRNAs), small noncoding RNAs, act as posttranscriptional gene expression modulators and have been implicated as key regulators in several physiological and pathological processes linked to CAD. Circulating miRNAs have been evaluated as promising novel biomarkers of CAD, acute coronary syndromes, and acute myocardial infarction, with prognostic implications. Several challenges related to technical aspects, miRNAs normalization, drugs interaction, and quality reporting of statistical multivariable analysis of the miRNAs observational studies remain unresolved. MicroRNA-based therapies in cardiovascular diseases are not ready yet for human trials but definitely appealing. Through this review we will provide clinicians with a concise overview of the pros and cons of microRNAs.

1. Introduction

Approximately every 42 seconds, a US American will suffer a heart attack. Cardiovascular and cerebrovascular diseases represent the leading cause of death worldwide, even if death rates have fallen from 1968 to the present [1]. Moreover, the lifetime risk for coronary heart disease varies drastically as a function of risk factor profile. With an optimal risk factor profile, lifetime risk for CHD is 3.6% for men and <1% for women; with ≥2 major risk factors, it is 37.5% for men and 18.3% for women [2]. Therefore, a correct identification of those individuals by specific biomarkers related to diagnosis, screening, staging, monitoring, surveillance, prognosis, and treatment selection would be of pivotal importance. Genetics, intermediate phenotype, life-style, and other environmental triggers are directly involved in the pathogenesis of coronary artery disease (CAD). The estimated heritability of CAD ranges from 30 to 60% [3, 4]. Recently, several studies highlighted that the genetics of CAD largely derives from the cumulative effect of multiple common risk alleles, emphasizing the individual but cumulative small effect size rather than rare variants with large effects on CAD risk. Despite this finding, there has been less success in understanding the function of the novel loci; in fact the majority of these loci are in noncoding regions of the genome [5]. Even if most of our genome does not encode for proteins and it is extensively transcribed anyway, generating non(protein) coding RNAs. Short noncoding RNAs of approximately 22–24 nucleotides, microRNAs, are widely recognized posttranscriptional gene regulator, while longer (>200 nucleotides) noncoding RNAs are now also recognized to play important roles in gene regulation and function [6]. MicroRNAs in cardiovascular disease are gaining momentum as possible novel biomarkers in the diagnosis and prognosis of coronary artery disease, acute coronary syndrome, and heart failure. Nowadays, diagnosis of acute coronary syndrome relies on symptoms, electrocardiogram abnormalities, and troponin quantification, with much interest in developing new rule-out and rule-in strategies or possible new promising biomarkers. In the literature, there is much ado about a possible clinical role of microRNAs in coronary heart disease. We aimed to review the pros and cons of microRNAs use in coronary heart disease applied to the clinical setting.

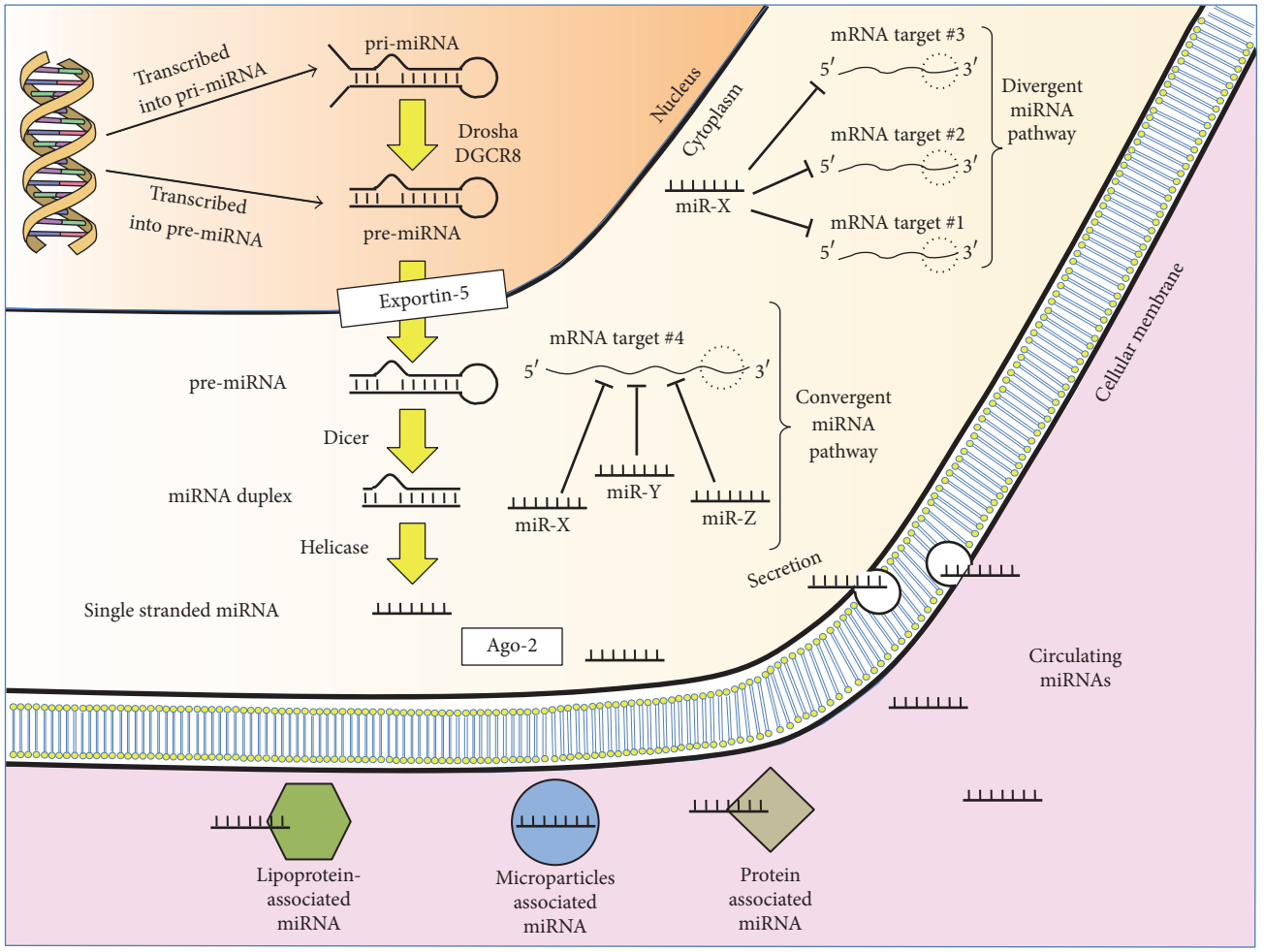

Figure 1: MicroRNAs biogenesis and function. In the nucleus, DNA is transcribed into pri-miRNA and then cleaved by Drosha to produce pre-miRNA in the canonical microRNA biogenesis pathway. Noncanonical biogenesis pathways exist. Pre-miRNA is then moved in the cytoplasm by Exportin-5 where another RNase III, Dicer cleaves it into a microRNA duplex, to finally obtain a single stranded microRNA. The microRNA can exercise his action internally or in a cell-to-cell interaction, through convergent or divergent microRNA pathways. Circulating microRNAs are usually associated with lipoprotein, protein, exosome, and microvesicles. See text for further details. Ago-2: Argonaute protein 2; miRNA: microRNA; mRNA: messenger RNA.

2. MicroRNAs

2.1. MicroRNA Biology. In 1993, Lee et al. [7] discovered that the *C. elegans* gene *lin-4* (a gene controlling the nematode larval development) did not encode for a protein but for small noncoding RNAs. A longer one (61 nucleotides) was then cleaved and folded in a stem-loop of 22 nucleotides. This *lin-4* derived RNAs had antisense complementarity to multiple sites in the 3′-UTR of the *lin-14* gene, with a final result of reducing the amount of LIN-14 protein, without changing the amount of *lin-14* messenger RNA (mRNA) [8]. This short lin-4 RNA is the founding member of the microRNAs family. The second member of this family, let-7, had to wait until the year 2000 to be described by Reinhart et al. [9]. Since then the miRNAs family has markedly expanded and more than 2000 different miRNAs sequences have been described and catalogued in miRBase [10]. MicroRNAs function as gene regulators acting on mRNAs translation, with inhibition of protein synthesis. Basically, different miRNAs may target a given mRNA in different binding sites (convergent microRNA pathway) or a single miRNA may target multiple different mRNAs (divergent miRNA pathway) [11]. There are specific types of software to predict which mRNAs may be the target of a specific miRNA (TargetScan, http://www.targetscan.org/; miRanda, http://www.microRNA.org/; TarBase (http://www.microrna.gr/tarbase).

MicroRNAs biogenesis is resumed in Figure 1. Briefly, in the canonical miRNAs biogenesis pathway, primary miRNAs (pri-miRNAs) of hundreds or thousands of nucleotides are synthesized from DNA by the enzyme RNA polymerase II in the nucleus. Pri-miRNAs, folded in the hairpin structure, are then cleaved by the ribonuclease III Drosha with the cofactor DGCR8, to form the microprocessor complex, producing the preliminary miRNAs (pre-miRNAs) of 70–100 nucleotides. The pre-miRNAs are transported into the cytoplasm by Exportin-5 where another ribonuclease III, Dicer, and its

cofactor TRBP, cleave them into the shorter, double stranded immature microRNAs. The miRNA-miRNA* duplex is then transferred to the Argonaute protein family (Ago) that undergoes conformational changes to allow binding of the miRNAs duplex. In the strand selection process, the passenger strand or miR* is discarded while the leading strand or miR is incorporated into the RISC (RNA-Induced Silencing Complex). Into the RISC, the miRNA presents the seed sequence at an interface where it can interact with a region of the mRNA within its 3′-UTR [12]. Other non-canonical miRNAs biogenesis pathways have been described [13].

2.2. Circulating MicroRNA. MicroRNAs can act intracellularly or can be actively secreted by cells and contribute to intercellular or cell-tissue communication [14]. Circulating microRNAs are stable despite the high extracellular RNase activity, due to their packaging in apoptotic bodies, microvesicles, and exosomes or association with lipoprotein, protein as the Argonaute family and other RNA binding proteins. Microvesicles and exosomes are fundamentally different, the first being smaller and heterogeneous in size, ranging from 100nm to 1μm, derived from the plasma membrane and released by budding and fission of the membrane, while the latter being formed intracellularly via endocytic invagination and then released into a multivesicular body [15]. Since the discovery in 2008 of miRNAs in blood [16], circulating miRNAs have been found in blood, urine, breast milk [17], saliva [18], tears, and other body fluids [19]; their potential use as serum biomarkers has become more appealing. Biomarkers should be divided into two different categories, depending on their possibility to change over time: genetic markers, stable over time, and dynamic markers, which may change mainly over time. A biomarker should be noninvasively obtained and have a high degree of sensitivity and specificity, permitting early diagnosis of disease. Moreover, a biomarker should have time-related changes in the disease course, a long half-life within the sample, allowing rapid and cost-effective laboratory detection. Some of these essential characteristics are shared by circulating miRNAs: their small size, a simple chemical composition, their high stability in boiling water, their resistance to extreme pH changes, prolonged room temperature stays or repeated freeze-thawing [19, 20], less complexity in comparison with proteins, and a cost-effective quantification by real-time polymerase chain reaction (qRT-PCR).

3. MicroRNAs in Coronary Heart Disease

3.1. MicroRNAs in Acute Coronary Syndrome. In 2010, five authors [21–25] independently reported a possible role for cardiomyocyte-enriched miRNAs in the diagnosis of acute myocardial infarction (AMI). Specifically, in these studies taken together, miR-1, miR-133a, miR-133b, miR-208, and miR-499 were found upregulated in plasma of AMI patients. Figure 2 resumes the potential miRNAs up- and down-regulated in AMI with diagnostic and prognostic implications. More than 30 studies analyzed the possible diagnostic

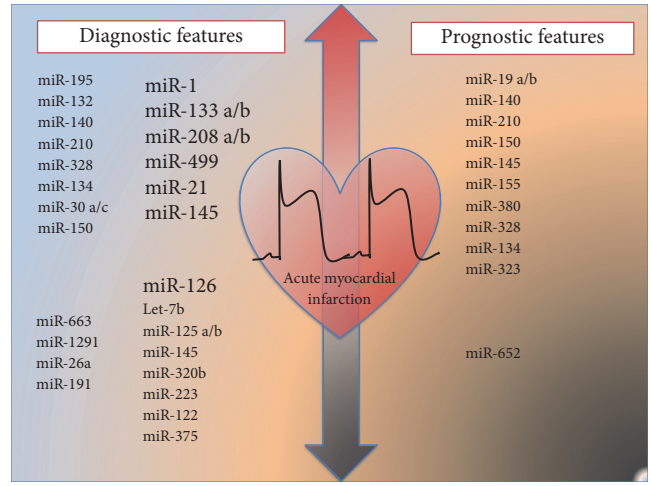

Figure 2: Circulating microRNAs associated with diagnostic and prognostic features in acute coronary syndrome (ACS). microRNAs in bigger font have been associated with ACS in more than one study. See Table 1 for further details.

microRNAs signature in AMI and other possible miRNA candidates have been proposed, but further validation studies are needed [26]. Years later, almost the same miRNAs are recognized as cardiac-enriched and proposed as possible biomarkers among several other miRNAs by 2 different authors. In 2014, a meta-analysis [27] of 19 studies evaluated the specificity and sensitivity of miR-1, miR-133a, miR-208b, and miR-499 in AMI. Cheng et al. concluded that miR-499 and miR-133a are possible biomarkers of AMI, showing a sensitivity of 0.88 (95% CI: 0.86–0.90; $p = 0.0000$); a specificity of 0.87 (95% CI: 0.84–0.90; $p = 0.0000$) and a sensitivity of 0.89 (95% CI: 0.83–0.94; $p = 0.0047$); a specificity of 0.87 (95% CI: 0.79–0.92; $p = 0.0262$), respectively. More recently, in a systematic review [28] the authors proposed that only cardiomyocyte-enriched miRNAS, miR-1, miR-133a/b, miR-145, miR-208a/b, and miR-499(a) in plasma and/or serum are potential biomarkers for the diagnosis of coronary heart disease.

Devaux et al. [29] presented the largest multicenter study on miRNAs in 1155 unselected patients with acute chest pain. miR-208b provided the highest diagnostic accuracy in AMI but still this was lower than that of the fourth-generation or high-sensitivity cardiac troponin T (cTnT). None of the six miRNAs provided added diagnostic value when combined with cTnT.

The prognostic role of miRNAs is encouraging. Few studies [29–34] have evaluated the role of miRNAs as prognostic biomarkers with controversial results; see Table 1 for further details. Very recently Karakas et al. [34] found for the first time that peripheral-blood miRNAs (miR-132, miR-140-3p, and miR-210) could predict CV mortality in a large cohort of ACS and stable CAD patients, while none of the cardiomyocyte-enriched miRNAs evaluated by Devaux et al. [29] predicted long-term mortality at 2-year follow-up, neither miR-208b nor miR-499 were significant predictors of mortality [33, 35]. Widera et al. [31, 34] found that miR-133a

Table 1: Selected studies on circulating microRNAs with prognostic implications after acute myocardial infarction. ACS: acute coronary syndrome; AMI: acute myocardial infarction; CAD: coronary artery disease; CV: cardiovascular; HF: heart failure; hsTnT: high-sensitivity troponin T; MACE: major adverse cardiac event.

Dysregulated miRNAs	Prognosis	Specimen	Normalization	Study population	Follow-up	References
↑ miR-132, miR-140-3p, miR-210	Predicted CV in ACS patients	Serum	*C. elegans* miR-39	430 ACS patients + 682 stable CAD patients	4 years	Karakas et al. 2016 [34]
↑ miR-208b	Predicted 30-day mortality with moderate accuracy	Plasma	*C. elegans* miRs	1155 chest pain patients	2 years	Devaux et al. 2015 [29]
↑ miR-145 on day 5	Predictive of MACE and CV death within 1 year after AMI	Serum	miR-16	246 STEMI patients	1 year	Dong et al. 2015 [64]
↑ miR- 208b, miR-499	Nonsignificant predictors	Plasma	*C. elegans* miRs	510 AMI patients (113 NSTEMI, 397 STEMI)	2–6 years	Goretti et al. 2013 [35]
↑ miR-155, miR-380	Predictive for cardiac death 1 year after AMI	Serum	—	26 patients who died of CV death within 1 year after AMI + 28 event-free AMI patients	1 year	Matsumoto et al. 2012 [32]
↑ miR-133a, miR-208b	miR-133a and miR-208b levels were significantly associated with the risk of death and lost their independent association with outcome upon further adjustment for hsTnT	Plasma	*C. elegans* cel-miR-54	444 ACS patients	6 months	Widera et al. 2011 [31]
↑ miR-208b, miR-499-5p	Equal to TnT for prognostic 30-day death after AMI	Plasma	miR-17	424 ACS patients	30 days	Gidlöf et al. 2013 [33]
↓ miR-652	Predictive of readmission to the hospital for heart failure within 5 years	Plasma	Synthetic UniSp4 and cDNA synthesis UniSp6	235 ACS patients + 116 healthy controls	5 years	Pilbrowa et al. 2014 [65]
↑ miR-133a	Associated with decreased myocardial salvage, larger infarcts, and more pronounced reperfusion injury but failed to prevent events	Serum	*C. elegans* miR-39	216 STEMI patients	6 months	Eitel et al. 2012 [66]
↑ miR-192, miR-194, miR-34a	Elevated by the early days after AMI in patients who experienced HF	Serum	U6 snRNA	21 AMI patients who developed HF within 1 year after AMI + 65 event-free AMI controls	1 year	Matsumoto et al. 2013 [36]

and miR- 208b levels were significantly associated with the risk of death in ACS patients, but in adjusted analysis their independent association with outcome was lost. Matsumoto et al. [32, 36] proposed 2 different sets of miRNAs with prognostic implications at 1-year follow-up post-AMI, but validation studies are needed for both.

3.2. Controversies in the Role of miRNAs as Biomarkers of AMI. The role of miRNAs has novel biomarkers in the early diagnosis of AMI is debated. The index test is high-sensitivity cardiac troponin, which is widely used in clinical practice and shows high accuracy in AMI diagnosis; therefore, it is very difficult for new biomarkers to demonstrate significant added value on top of cardiac troponins. Moreover, the 3rd universal definition of AMI relies on symptoms and detection of troponin-positive myocardial necrosis, even if the unspecific elevation of troponin levels can be present in case of nonischemic heart failure (HF), renal failure, myocarditis, arrhythmias, and pulmonary embolism due to myocardial injury [37]. Wang et al. [25] reported that, in AMI patients, miR-208 levels were not altered by chronic kidney disease or trauma, as it happens for troponins. Actually miRNAs can reduce this gap and provide additional accuracy in the diagnosis of AMI, as some miRNAs became detectable when initial troponin was still negative or within 3h of symptom onset [38]. *De iure* miRNAs become detectable earlier than high-sensitivity troponin, theoretically allowing a faster rule-in/rule-out of chest pain patients; one of the major limits of cTnT is that multiple dosage at different time is needed and patients are ordered to stay in the emergency room for 3–6 h after arrival. *De facto* measurement of circulating miRNAs requires qRT-PCR, which is a time-consuming technique, in comparison with detection of hs-cTnT (approximately 30 min) and the 2015 ESC guidelines recommend the use of a rapid rule-out protocol (0 h and 1 h or 0 and 3 h) when hs-cTnT is available [39]. The use of qRT-PCR is currently the limiting factor in terms of rapid detection of circulating miRNAs. In the future, the availability of newer, faster, and cost-effective techniques may overcome this limit.

3.3. MicroRNAs in Coronary Artery Disease. The ability to distinguish stable from unstable angina pectoris patients would be a great advance in CAD management, but this promise is far from being fulfilled, as concluded by D'Alessandra et al. [40]. Several miRNAs, as cardiomyocyte-enriched (miR-133, miR-208a) [41], endothelial cell-enriched (miR-126, miR-17-92a cluster), vascular smooth cell (miR-143/145) and inflammatory cell-enriched (miR-155), and platelet-enriched (miR-199a) miRNAs, were associated with CAD, while lipometabolism-related miR-122 and miR-370 increased as the severity of CAD quantified by the Gensini score increased [42]. Previously, miR-126 has been proposed as a prognostic marker of incident myocardial infarction in the general population [43], result partially confirmed by Jansen et al. [44] who reported that only microvesicles-associated miR-126 and miR-199a predict the occurrence of CV events in patients with stable CAD. A more comprehensive review has been recently published [45].

4. Technical Aspects of miRNAs Quantification

The sensible differences and heterogeneous results reported in ACS and CAD studies can be partly explained by some technical aspects and drugs interaction. Quantification of miRNAs transcripts by qRT-PCR implicates data normalization with endogenous and exogenous reference genes for data correction. Data from qRT-PCR can be analyzed using absolute or relative quantification. Absolute quantification defines expression levels in absolute numbers of copies by relaying the PCR signal to a standard curve. Relative quantification determines fold changes in expression between two samples, normalizing the gene of interest for a housekeeping gene in the same sample to obtain a fold change. One of the most frequently used normalizers is the small noncoding RNA RNU6, which is not a miRNA and could not perfectly reflect the miRNAs biochemical characteristics. miR-16 is another frequently used normalizer because it is highly expressed and relatively invariant. The choice of the reference gene can be challenging as an optimal normalization strategy is missing. Consequently, the choice of which miRNAs should be used as internal controls for circulating miRNAs assessment could lead to ambiguous data interpretation, misleading conclusions, and erroneous biological predicted effect, impairing comparison between studies and meta-analysis of data. The use of more than 1 reference gene increases the accuracy of quantification; for example, the combined use of miR-16 and other miRNAs could reduce the potential bias compared to the use of a single reference gene [46]. Some authors stated that, in the lack of a shared housekeeping miRNA, miRNAs expressions do not require an internal control and could be normalized to serum volume [47]. However, this strategy has been demonstrated to increase the risk of bias and should be avoided. In addition, while searching for the ideal normalization gene candidate, it is pivotal to apply standardization across laboratories for sample preservation, storage, and stability.

Another potentially confounding factor is drug administration. Statins [42], anticoagulation [48], and antiplatelet drugs [49] can affect quantification of miRNAs in blood samples and must be taken into account when assessing circulating miRNAs [50]. To overcome the potential confounding effect of heparin, Kaudewitz et al. suggested normalizing with exogenous *C. elegans* spike-in control [51]. Other options to treat plasma from patients subjected to heparin treatment include digestion with heparinase on purified RNA rather than plasma, optimization of the starting plasma volume, and enrichment of miRNA on silica [52, 53]. To successfully translate miRNA signature in clinical practice it is mandatory to develop and apply a standardization of the operative procedures related to circulating miRNAs analysis. Standardization needs to be applied at several stages, from blood withdrawal to plasma/serum centrifugation, to sample collection and banking, and to RNA extraction and microRNAs quantification, in order to dramatically reduce interlaboratory differences that could generate huge incoherencies in miRNAs analyses. Consequently, bias in the selection, extraction, and quantification of miRNAs generating unexplained

Table 2: Items reviewed on observational studies assessing the value of microRNAs as potential biomarkers for coronary artery disease and acute coronary syndrome. Table adapted by authors from [55].

Item	Issue	Question
(1)	Model assumption and goodness-of-fit	How far away from the data is the selected model?
(2)	Interaction analysis	Is there any potential variable that can modify the estimated effect?
(3)	Sensitivity analysis	Are the findings sufficiently robust, considering the process used to obtain them?
(4)	Crude and adjusted effect estimate	How much does the studied effect change when other variables are taken into account?
(5)	More than one adjusted model specified	Does the estimated effect differ between the different adjusted models, settings, specifications, and so forth?

contrasting results is widespread in most studies and represent a major limitation to perform a meta-analysis.

5. Quality Reporting in Circulating MicroRNAs Observational Studies in Coronary Heart Disease

Several authors reported that the biggest limitation for use of miRNAs as biomarkers is the small sample size of published studies [48, 54]. Not only small sample size, but also the quality of reporting of observational studies is a major issue, due to the lack of randomized double blind trials. Controlling for already mentioned confounders is a crucial step in microRNAs observational studies, to avoid misleading conclusion. To overcome this problem, the use of multivariable models as statistical adjustment techniques is widely encouraged and the validation of assumption of the multivariable regression models should be clearly stated in the methodology. To our knowledge, a quality report on statistical methodology in circulating miRNAs studies has not yet been performed. We reviewed the quality of statistical reporting of 56 studies (see Supplementary Figure S1 and Table S1 for included studies in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2150763) on circulating miRNAs in coronary heart disease (ACS, AMI, and CAD). A list of the Real et al. items reviewed in full-text studies is presented in Table 2, based on Real et al. methods [55]. See Supplementary Materials for complete methods and statistical analysis. Results are resumed in Table 3. The large majority of studies are in fact cohort studies of small sample size (median size 115 patients). Of note, significant differences exist between small (<100 patients) and large sample size (>500 patients) studies in terms of quality reporting of multivariable regression models. A multiple regression was run to predict a quality score >2 from adopted model, journal impact factor, citation/year, and sample size. Among these variables, only sample size statistically significantly predicted quality score, $F(5, 50) = 22.201$, $p < 0.0001$, $R^2 = .689$. The totality of larger sample size studies scored at least 3 over 5 checked items, demonstrating a solid methodology and control for confounders. In fact, some miRNAs lost statistical significance when adjusted for confounders [29, 34, 43]. It is highly possible that among the authors of large sample size study a methodologist is included. Small sample size studies without adjustment for confounders of the results contribute to increase heterogeneity and introduce possible bias in the literature. No significant differences exist in terms of article citations per year; highly cited articles can have a robust or a weak methodology. Obviously the first reports were small sample size studies but great breakthroughs in microRNAs biology and function were therefore highly cited. Studies with weak methodology can present contrasting results and then be cited in contrast to more robust studies, creating confusion. Nevertheless, the journal impact factor has definitely a role in assessing the methodological quality of the study and even if it does not reach the full significance in our results, the trend is in favor of a positive correlation between impact factor and high methodological score.

6. MicroRNA-Based Therapeutics

Up-to-date microRNAs-based therapies are in their infancy, thus experimental and animal studies are in favor of a potential role in the treatment of CV diseases. In nonhuman primates, inhibition of miR-33a and miR-33b by an anti-miRNA oligonucleotide increased hepatic expression of ABCA1, a key regulator of high density lipoprotein (HDL) biogenesis, and induced a sustained increase in plasma HDL levels over 12 weeks, with reduction of very low density lipoprotein (VLDL) levels [56]. Another study assessed the role of locked nucleic acid-modified antisense miR-92a (LNA-92a) in a model of ischemia/reperfusion injury in pigs and revealed cell-protective, proangiogenic, and anti-inflammatory effects of LNA-92a with reduction of infarct size and improved recovery of cardiac function [57]. Unfortunately these promising results have not yet progressed to human trials. After the seminal studies on miR-21 by Thum [58], a key target in CV diseases would be reduction/inhibition of myocardial fibrosis associated with postischemic cardiopathy, drug-induced or primitive cardiomyopathies [59], but the question is far from being resolved yet [60, 61]. In other fields of medicine miRNA-based therapies are a step forward. In patients with chronic hepatitis C, subcutaneous administration of an antisense oligonucleotide for miR-122 led to successful results with negligible side effects in phases 1 and 2a trial [62] and at long-term follow-up [63].

Table 3: Frequency of application of multivariable regression models, based on study features, of the observational studies assessing the value of microRNAs as potential biomarkers for coronary artery disease and acute coronary syndrome.

Variable	Median (1st quartile; 3rd quartile)	Category	*N*	Model assumption	Interaction analysis	Sensitivity analysis	Crude and adjusted effect estimate	More than one adjusted model specified	Reporting at least > 2 items
Articles	—		56	29 (52%)	32 (57%)	30 (54%)	14 (25%)	19 (34%)	34 (61%)
		95% CI		39–64%	44–69%	41–66%	15–38%	23–47%	48–72%
Publication year	2013 (2012; 2014)			$p = 0.418$	$p = 0.941$	$p = 0.596$	$p = 0.139$	$p = 0.869$	$p = 0.806$
		2010-2011	12	7 (58%)	6 (50%)	7 (58%)	0 (0%)	3 (25%)	3 (25%)
		2012-2013	17	7 (41%)	10 (59%)	11 (65%)	5 (29%)	6 (35%)	6 (35%)
		2014-2015	25	13 (52%)	15 (60%)	11 (44%)	8 (32%)	9 (36%)	10 (40%)
		2016	2	2 (100%)	1 (50%)	1 (50%)	1 (50%)	1 (50%)	1 (50%)
Sample size	115 (58; 312)			$p = 0.129$	$p = 0.138$	$p = 0.054$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$
		<100	30	13 (43%)	14 (47%)	12 (40%)	1 (3%)	4 (13%)	4 (13%)
		101–500	19	10 (53%)	12 (63%)	12 (63%)	7 (37%)	8 (42%)	9 (47%)
		>501	7	6 (86%)	6 (86%)	6 (86%)	6 (86%)	7 (100%)	7 (100%)
Design	—				$p = 0.616$	$p = 0.329$	$p = 0.774$	$p = 0.427$	$p = 0.557$
		Cross-Sectional	11	7 (64%)	5 (45%)	8 (73%)	3 (27%)	5 (45%)	5 (45%)
		Cohort	36	17 (47%)	21 (58%)	17 (47%)	8 (22%)	10 (28%)	11 (31%)
		Case-Studies	9	5 (56%)	6 (67%)	5 (56%)	3 (33%)	4 (44%)	4 (44%)
Journal impact factor	3.4 (1.8; 5.8)			$p = 0.327$	$\mathbf{p < 0.001}$	$p = 0.015$	$p = 0.111$	$p = 0.05$	$p = 0.034$
		<3	27	12 (44%)	8 (30%)	10 (37%)	4 (15%)	5 (18%)	5 (18%)
		3–6	16	8 (50%)	13 (81%)	13 (81%)	4 (35%)	7 (44%)	8 (50%)
		>6.01	13	9 (69%)	11 (85%)	7 (54%)	6 (46%)	7 (54%)	7 (54%)
Yearly Scopus citations	8 (3; 16)			$p = 0.538$	$p = 0.635$	$p = 0.203$	$p = 0.918$	$p = 0.161$	$p = 0.283$
		<7	24	11 (46%)	12 (50%)	10 (42%)	6 (25%)	5 (21%)	6 (25%)
		7–16	18	9 (50%)	11 (61%)	10 (56%)	5 (28%)	7 (39%)	7 (39%)
		>16.1	14	9 (64%)	9 (64%)	10 (54%)	3 (21%)	7 (50%)	7 (50%)

7. Conclusion

Translational research represents a stem of scientific research that helps to make findings from basic science useful for practical applications that enhance human health and well-being. Deeply established on multidisciplinary collaboration, translational research has the enormous potential to move applied science forward. Accordingly, in the cardiovascular system, miRNAs fine-tune complex molecular signaling networks by acting on key target proteins involved in a variety of pathways and cellular processes. Therefore, in the past decade, several studies emphasized the importance of miRNAs as diagnostic and prognostic role in cardiovascular disease and the road travelled so far seems promising for a specific role in coronary heart disease. At present, circulating miRNAs have not entered yet the clinical arena, due to contrasting results, possible confounding factors, presence of small or moderately sized studies of different methodology, sometimes challenging each other, technological requirements, and unstandardized normalization. This complex scenario, in which bordering results contradict themselves, may push researchers, clinicians, and also patients in different directions providing dissimilar effect estimates with mixed results and with benefits ranging from absent to transient and, at most, marginal. In the future, the role of long noncoding RNAs may add novel insight into the posttranscriptional regulation changing the way with which investigators identify novel signal transduction pathways and functional cross-talks developing new therapeutic strategies and micro-RNA based therapies might make the way for human trials with important therapeutic implications. Clinicians must be aware of the pros and cons of microRNAs advent and read critically the fore coming literature.

Abbreviations

ACS:	Acute coronary syndrome
AMI:	Acute myocardial infarction
CAD:	Coronary artery disease
CV:	Cardiovascular
DGCR8:	Di George syndrome critical region 8
HDL:	High density lipoprotein
hs-cTNT:	High-sensitivity cardiac troponin T
LNA:	Locked nucleic acid
miRNA:	MicroRNA
mRNA:	Messenger RNA
qRT-PCR:	Quantitative real-time polymerase chain reaction
TRBP:	TAR RNA-binding protein
VLDL:	Very low density lipoprotein.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Role of miRNAs in Epicardial Adipose Tissue in CAD Patients with T2DM

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Background. Epicardial adipose tissue (EAT) is identified as an atypical fat depot surrounding the heart with a putative role in the involvement of metabolic disorders, including obesity, type-2 diabetes mellitus, and atherosclerosis. We profiled miRNAs in EAT of metabolic patients with coronary artery disease (CAD) and type-2 diabetes mellitus (T2DM) versus metabolically healthy patients by microarray. Compared to metabolically healthy patients, we identified forty-two miRNAs that are differentially expressed in patients with CAD and T2DM from Xinjiang, China. Eleven miRNAs were selected as potential novel miRNAs according to *P* value and fold change. Then the potential novel miRNAs targeted genes were predicted via TargetScan, PicTar, and miRTarbase, and the function of the target genes was predicted via Gene Ontology (GO) analysis while the enriched KEGG pathway analyses of the miRNAs targeted genes were performed by bioinformatics software DAVID. Then protein-protein interaction networks of the targeted gene were conducted by online software STRING. Finally, using microarray, bioinformatics approaches revealed the possible molecular mechanisms pathogenesis of CAD and T2DM. A total of 11 differentially expressed miRNAs were identified and among them, hsa-miR-4687-3p drew specific attention. Bioinformatics analysis revealed that insulin signaling pathway is the central way involved in the progression of metabolic disorders. *Conclusions*. The current findings support the fact that miRNAs are involved in the pathogenesis of metabolic disorders in EAT of CAD patients with T2DM, and validation of the results of these miRNAs by independent and prospective study is certainly warranted.

1. Introduction

Coronary artery disease (CAD) remains one of the most common causes of morbidity and mortality in diabetic patients [1]. Thus improving the understanding of the etiology associated with CAD is highly important. Epicardial adipose tissue (EAT) is suggested to play an important role in the progression of metabolic syndrome [2]. Several findings implicate that EAT thickness may be a useful indicator for T2DM and obesity [2, 3]. Studies have shown that EAT generates several bioactive molecules, such as anti- and proinflammatory mediators and cytokines [4], which may significantly enhance paracrine effects on cardiac function or produce a variety of effects that affect many physiological processes [5]. Nowadays, beside the main factors including obesity, hypertension, and dyslipidemia, novel risk factors such as chronic low-grade inflammation, oxidative stress, and endothelial dysfunction are accepted as the decisive factors to highlight this increased cardiovascular risk in human beings [6, 7]. Experimental and clinical studies have suggested that EAT may cause CAD [8]. EAT, visceral fat depot of the heart, was found to be associated with CAD, T2DM, and other metabolic disorders [6, 9, 10]. EATs are metabolically active visceral fat deposits found around the heart, between the pericardium and myocardium [11], which are strongly associated with cardiovascular diseases (CVD) including CAD and the development of cardiac arrhythmias, predominantly due to the secretion of bioactive mediators and cytokines [12]. T2DM plays a key role in the development of CVD. EAT has great interplay with diabetic patients and has potential to influence CVD. Owing to its close proximity to the heart

and coronary vasculature, EAT exerts a direct metabolic impact by secreting free fatty acids and proinflammatory factors and decreased anti-inflammatory adipokines, which promote CVD locally [9]. MicroRNAs (miRNAs) are a class of about 21–25 nucleotides in length and small noncoding RNA molecules with essential roles, of which any alteration leads to several conditions. They were first identified in 1993, and the term microRNA was created in 2001 [13]. The main functions of miRNAs are to downregulate the target gene expression in translational repression and cleavage of mRNA and in a wide range of biological processes [13]. Decisive regulatory functions exhibited by the miRNA are associated with various human diseases such as human cancer and heart disease. In addition to the link with cancer, microRNAs play a vital role in the control of cardiac-related diseases. For multiple forms of heart disease, including ventricular wall, maintenance of cardiac rhythm myocyte growth, and contractility, the misexpressions of miRNAs were shown to be necessary. However, in the literature, data regarding the relationship between miRNAs and EAT in CAD patients T2DM is scant. Hence, we aimed to investigate the role of miRNAs in EAT. We also sought to predict the targets of novel miRNAs in EAT in patients compared with the results that are obtained from control subjects [6]. The current study was carried out to assess the changes in the EAT levels of miRNAs in subjects suffering from T2DM and CAD compared to healthy control ones. In addition, bioinformatics analyses were carried out in order to find out how these possible miRNAs are associated with the incidence and pathogenesis of CAD on top of T2DM. In the present study, we depicted comparative miRNAs in "metabolically healthy" patients without metabolic disorders and in metabolic patients with CAD and T2DM. This strategy allowed us to identify a set of miRNAs characterizing EAT in health and disease as well as potential novel biological processes characterizing EAT in CAD patients with T2DM.

2. Methods

2.1. Subjects. EAT samples were taken from 10 subjects of both genders, in the Department of Thoracic and Cardiovascular Surgery, the First Affiliated Hospital of Shihezi University School of Medicine, in which levels of miRNAs expression in EAT (using microarray) and routine parameters were measured. Subjects were divided into two groups, 5 in each group as follows: patients with T2DM and CAD and metabolically healthy control subjects without T2DM and CAD. Ten Asian patients were recruited, 5 underwent cardiac valve surgery (no evidences of CAD/T2DM/carotid atherosclerosis/metabolic syndrome), and 5 underwent coronary artery bypass graft surgery (CAD + T2DM group). The study protocol was approved by the Medical Ethics Committee of Shihezi University (School of Medicine, Xinjiang, China). Written informed consent was obtained from all subjects included in the study. This was a cross-sectional study and a review of medical records (including information on sex, age, height, weight, medications, disease duration, smoking, and history of other diseases) was undertaken. Control subjects were chosen from metabolically healthy individuals according to NCEP ATP III Metabolic syndrome criteria (two or less metabolic criteria; TG $\geq$ 1.7 mmol/L, blood pressure $\geq$ 130/85 mmHg, glucose $\geq$ 5.6 mmol/L, HDL-C: men $<$ 1.03 mmol/L and women $<$ 1.30 mmol/L, and waist circumference: men $<$ 102 cm and women $<$ 88 cm) as previous said [6, 14]. miRNAs microarray expression analyses were conducted on RNA extracted from perivascular EAT using the Human-MicroRNA Expression Kits.

2.2. RNA Extraction and Purification. Total RNA, including the miRNAs, was extracted from the EAT and purified using *mir*Vana™ miRNA Isolation Kit (Cat. number AM1561, Ambion, Austin, TX, US), following the manufacturer's instructions, and checked for a RNA integrity number (RIN) to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). RIN $\geq$ 6.0 and 28S/18S $\geq$ 0.7 were used for the miRNA array analysis.

2.3. RNA Labeling. miRNA molecular in total RNA was labeled by miRNA Complete Labeling and Hyb Kit (Cat. number 5190-0456, Agilent Technologies, Santa Clara, CA, US) following the manufacturer's instructions, labeling section.

2.4. Array Hybridization. MiRNA microarray assays were performed using the Agilent Human miRNA (8 $*$ 60 K) V21.0 microarray platform (design ID: 70156) at Shanghai Biotechnology Co., Ltd. (Shanghai, China). Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit (Cat. number 5190-0456, Agilent Technologies, Santa Clara, CA, US) in hybridization oven (Cat. number G2545A, Agilent Technologies, Santa Clara, CA, US) at 55°C, 20 rpm for 20 hours according to the manufacturer's instructions, hybridization section. After hybridization, slides were washed in staining dishes (Cat. number 121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat. number 5188-5327, Agilent Technologies, Santa Clara, CA, US).

2.5. Data Acquisition. Slides were scanned by Agilent Microarray Scanner (Cat. number G2565CA, Agilent Technologies, Santa Clara, CA, US) and Feature Extraction software 10.7 (Agilent Technologies, Santa Clara, CA, US) with default settings. Raw data were normalized by Quantile algorithm, Gene Spring Software 12.6 (Agilent Technologies, Santa Clara, CA, US).

2.6. Differential miRNAs Targeted Gene Prediction. The differential miRNAs targets predicted by computer-aided algorithms were obtained from TargetScan, PicTar, and miRBase targets [15]. More detailed information can be acquired from online software (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi; http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl; http://www.targetscan.org/).

2.7. The Interaction Network and Signaling Pathway Analysis of Differential microRNA and mRNA. DAVID [16–18], a bioinformatics analysis software, is used for the analysis of the enriched KEGG (Kyoto Encyclopedia of Genes and

Table 1: Baseline characteristics of patients.

	Non-CAD + T2DM ($n = 5$)	CAD + T2DM ($n = 5$)	P value
Age (years)	61.8 ± 5.2	54.6 ± 7.0	0.392
BMI (kg/m^2)	29.47 ± 5.83	28.63 ± 4.26	0.213
WC (cm)	99.64 ± 10.67	101.23 ± 10.34	0.492
Waist-hip ratio	0.96 ± 0.14	0.99 ± 0.09	<0.001
FPG (mg/dL)	106.5 (98.5–115.5)	116.0 (100.5–138.0)	0.103
Total cholesterol	166.48 ± 37.10	159.55 ± 39.41	0.335
LDL-C (mg/dL)	93.51 ± 26.59	98.44 ± 23.14	<0.01
HDL-C (mg/dL)	40.66 ± 10.08	34.39 ± 9.64	<0.01
Triglyceride (mg/dL)	139 (105.5–223.5)	154 (106.0–234.0)	0.276
hsCRP (mg/dL)	1.03 ± 2.31	3.15 ± 5.22	<0.001
Adiponectin (μg/mL)	12.37 ± 6.55	9.16 ± 4.78	<0.05
Fibrinogen (mg/dL)	523.0 (453.0–638.0)	603.5 (510.5–766.5)	<0.01

FPG: fasting plasma glucose, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, and hsCRP: hypersensitive C reactive protein. Adiponectin, hsCRP, and fibrinogen indicate systemic inflammation.

Genomes) signaling pathway analysis for the interactions between microRNAs and mRNAs (http://david.abcc.ncifcrf.gov/). Online software Gene Ontology (http://geneontology.org/) was employed to perform GO enrichment analysis [19, 20]. The microRNA and mRNA of differential expression in patients were uploaded to DAVID and Gene Ontology for analysis.

2.8. Protein-Protein Interactions (PPI) Network Analysis. A number of abnormal mRNAs were found in the interaction analysis between miRNAs and mRNA. Therefore, to further understand the function of microRNA in the network, the PPI analysis was performed in the protein products of mRNAs to find out the key proteins. The selected targeted genes were put into the STRING (Search Tool for the Retrieval of Interacting Genes) database (http://string-db.org/), a metaresource that collects most of the available information on protein-protein associations and scores and weights it and augments it with predicted interactions and with the results of automatic opuses-mining searches to match the interactions of proteins [21, 22].

2.9. Statistical Analysis. Descriptive statistics for each variable were determined. Results for continuous variables were demonstrated as mean ± standard deviation. Statistical significant difference between the groups was determined by the chi-square test for categorical variables and unpaired Student's t-test for continuous variables. Differentially expressed targeted genes were studied using bioinformatics analysis and statistical analysis allowed algorithm of the selected software.

3. Results

3.1. Baseline Characteristics of Patients. The baseline characteristics of 5 patients and 5 control subjects were shown in Table 1. There were no differences with respect to the following variables between patients and control subjects, age, gender, waist circumference (WC), systolic blood pressure (SBP), diastolic blood pressure (DBP), and body mass index (BMI). Compared to "controls," CAD patients with T2DM were characterized by significantly increased waist-hip ratio, LDL-C, and systemic inflammation, while HDL-C was decreased.

3.2. Data of Microarray. In order to measure the miRNAs expression patterns that characterize EAT in CAD patients with T2DM from EAT in control group, we used the whole-genome miRNAs microarrays. Overall, the resulting signal intensity of miRNAs genes is statistically different at the Wilcoxon signed-rank test in EAT in both groups, thus underscoring the profound diversity of EAT. Unsupervised hierarchical clustering was presented in Figure 1. Compared to metabolically healthy patients, we identified forty-two miRNAs that are differentially expressed in patients with CAD and T2DM (26 downregulated; 16 upregulated, data was not shown). Eleven miRNAs were selected as potential novel miRNAs according to P value and fold change (Table 2: 6 were significantly downregulated; 5 were significantly upregulated in both subgroups of patients with $P < 0.05$; fold change > 2 times).

3.3. Results of Bioinformatics Analyses. To depict the possible role of miRNAs in EAT, we selected these miRNAs as potential novel biomarkers that were significantly different (patients versus controls) in the overall population ($P < 0.05$; fold change > 2). To provide a framework for interpretation of our results, we then functionally clustered significant biological pathways using the bioinformatics analyses.

3.3.1. Bioinformatics Analyses of miRNAs Targeted Genes. In order to investigate the possible regulation mechanisms of miRNAs in the process of CAD complicated with T2DM, we first utilized three online bioinformatics databases (TargetScan, PicTar, and miRBase targets) to select plausible targets and validated targets of miRNAs and finally obtained target genes for the following analysis; then we analyzed biological processes, molecular functions, and cellular components through Gene Ontology and enriched KEGG pathways by DAVID. The results showed that the predicted

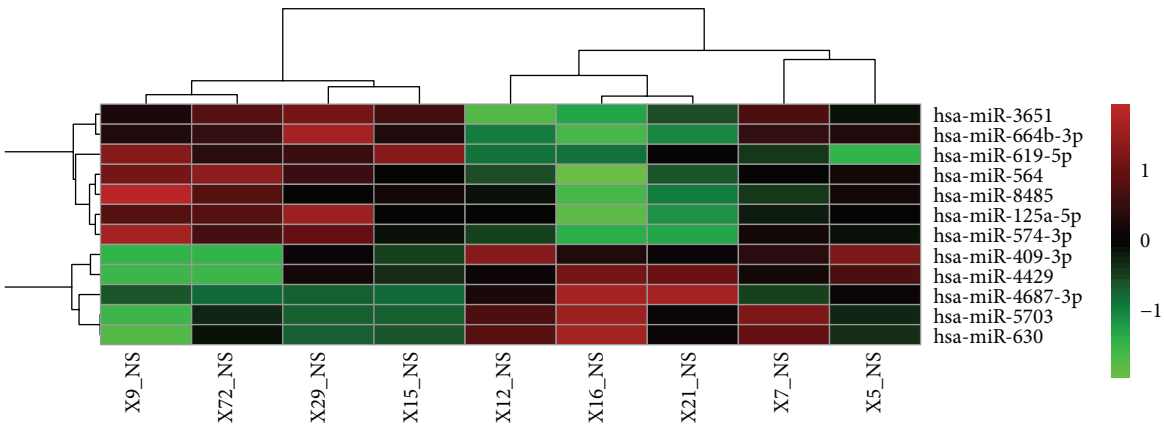


Figure 1: Unsupervised hierarchical clustering (heat map). Heat map generated by hierarchical clustering for differentially expressed miRNAs in the EAT from CAD with T2DM patients versus control subjects. Hierarchical clustering for differentially expressed miRNAs in CAD with T2DM ($n = 4$) versus control ($n = 5$) ($P < 0.05$ and fold change > 2 times). Columns display the clustering of EAT samples; rows show the clustering of genes. The expression intensity of each miRNA in each sample varies from red to green, which indicates relative high or low expression, respectively. Expression clusters representing different patterns of upregulation to downregulation are depicted on the side of figure.

Table 2: Disregulated miRNAs (CAD + T2DM versus control).

miRNA	P value	Fold change
hsa-miR-4429	0.04437405	0.16922769
hsa-miR-409-3p	0.012379586	0.184890055
hsa-miR-6802-5p	0.047496135	0.305662149
hsa-miR-5703	0.008804377	0.40973895
hsa-miR-630	0.020813173	0.43854972
hsa-miR-4687-3p	0.034377945	0.44430727
hsa-miR-3651	0.04317499	2.063333894
hsa-miR-574-3p	0.025198143	2.156938357
hsa-miR-619-5p	0.00179165	2.179715733
hsa-miR-664b-3p	0.04815202	2.325766896
hsa-miR-146b-5p	0.044983658	2.450112684

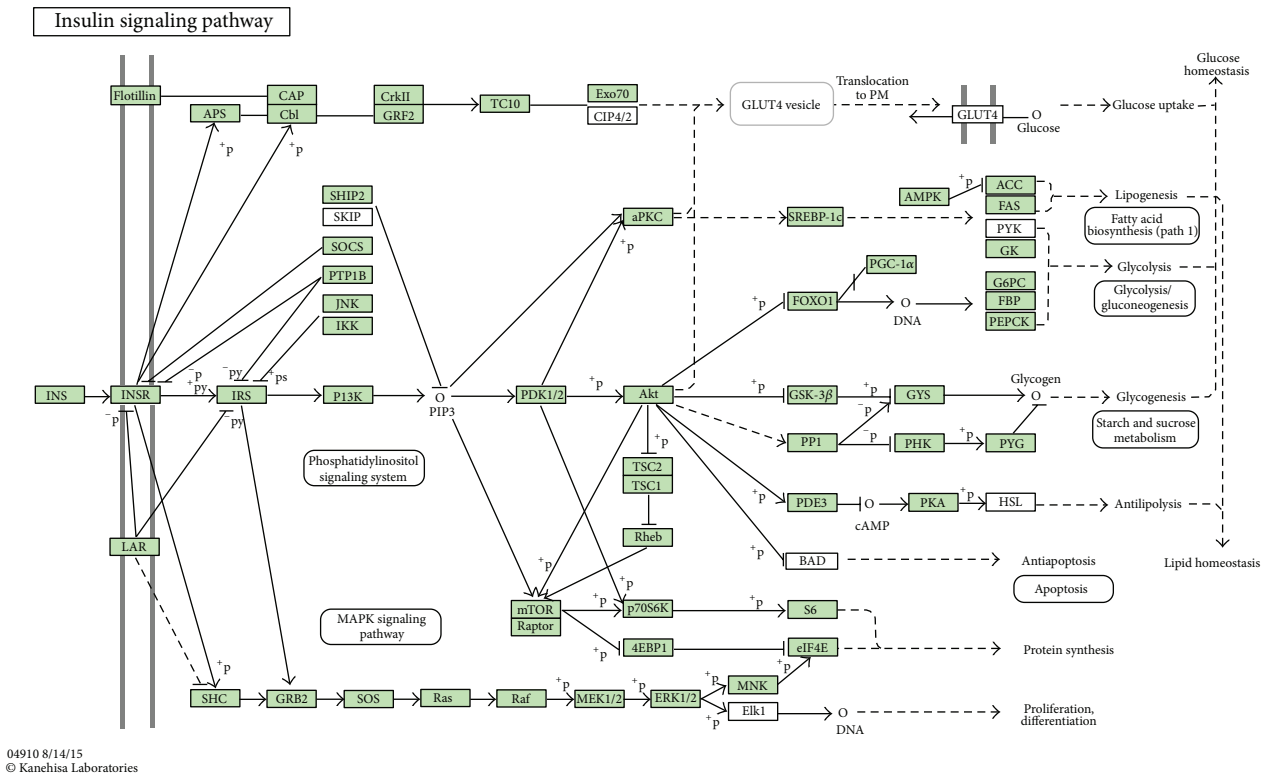
Lists of the deregulated miRNAs between CAD + T2DM and control. Fold change < 0.5 indicated downregulation significantly and fold change > 2 indicated upregulation significantly.

target genes mainly are enriched in the following biological processes: detection of chemical stimulus involved in sensory perception of smell, G-protein coupled receptor signaling pathway, translation, axon guidance, protein phosphorylation, and so forth ($P < 0.001$), they mainly are enriched in the following molecular functions: G-protein coupled receptor activity, olfactory receptor activity, protein binding, structural constituent of ribosome, and so forth ($P < 0.001$), and significant cellular components were extracellular region and Golgi apparatus ($P < 0.001$). Enriched KEGG pathways by DAVID displayed in KEGG pathway database showed that the predicted target genes of miRNAs were significantly enriched in the insulin signaling pathway (Figure 2), adipocytokine signaling pathway, MAPK signaling pathway, FoxO signaling pathway, and other signaling pathways ($P < 0.05$).

3.3.2. Protein-Protein Interaction (PPI) Network. We analyzed the protein-protein interaction network of selecting miRNAs target genes using STRING 10 and removed the target genes which were linked to lax isolated nodes through data analysis; the result showed that the interaction existed in total 148 proteins targeted by the predicted genes, which together formed the target gene interaction network. The network consists of 148 nodes which represent 148 proteins and many lines with different colors that represent the types of evidence for the association. From the result we can see that PDPK1, PIK3R3, PPP1R3B, PRKAR1A, SOCS3, SREBF1, PPARGC1A, SHC4, MAPK1, GRB2, and MKNK2 played key roles in maintaining stability in the network, especially PIK3R3, MAPK1, and GRB2 whose connections were very close, so the protein encoded by them may be important downstream target proteins (Figure 3).

4. Discussion

Better understanding of the biological characteristics can provide vital theoretical basis for the prevention and treatment of disease. The aim of this study was to investigate the profiles of miRNAs and the interaction network of novel microRNA and mRNA as well as related signaling pathway in EAT through analyzing the expression profile of microRNA and mRNA to provide novel insights in the biological characteristics in CAD patients with T2DM. According to newly added studies, it is clear that miRNAs are involved in the regulation of metabolic and inflammation functions, and the alterations in CAD and T2DM diseases have been analyzed through the identification and evaluation of miRNAs profiles in patients as in animal models [13, 23, 24]. microRNAs are key components of many cellular processes. Different studies have demonstrated that miRNA expression is tissue-specific, tightly regulated during embryogenesis, and overexpressed/underexpressed in many diseases,



+ps: serine phosphorylation
+py: tyrosine phosphorylation
−py: tyrosine dephosphorylation

Figure 2: Insulin signaling pathway. ERK1/2(MAPK1), GRB2, PKA (PRKAR), P13K (PIK3), and PDK1/2 (PDPK1) are the main target genes of differential miRNAs.

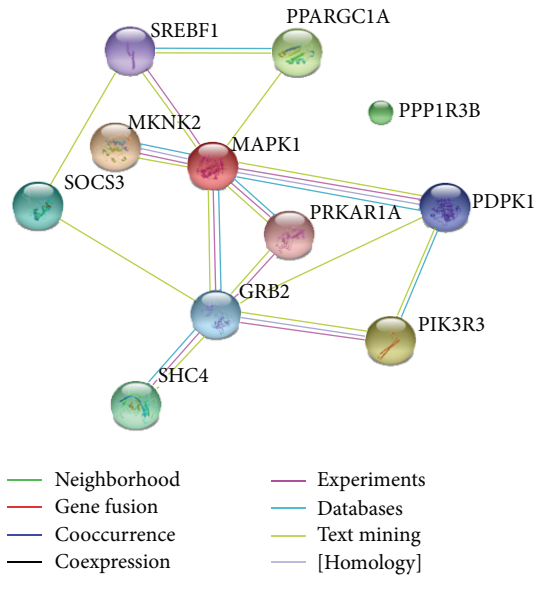


Figure 3: Protein-protein interaction (PPI) network of target proteins of the selecting miRNAs.

including CAD and T2DM pathologies [25]. They are easily detected in a quantitative way by real-time polymerase chain reaction (qRT-PCR) or microarrays and by other less frequently used identification methods, such as PCR-based restriction fragment length polymorphisms (PCR-RLFP), traditional northern blotting, direct sequencing using next generation sequencing (NGS), and platforms ligation based measurement [26]. Risk prediction for T2DM and CVD remains suboptimal even after the introduction of global risk assessment by various methods. This has prompted the search for additional biomarkers [27]. EAT is a source of several inflammatory mediators in high-risk cardiac patients [4]. Adipose tissue may function as an endocrine organ that contributes to an inflammatory burden in patients at risk of CVD. Obesity, adiposopathy, and insulin resistance induce EAT enlargement, inflammation, and dysfunction and trigger CAD [10]. EAT is an atypical fat depot surrounding the heart with a putative role in the development of atherosclerosis [28]. Given the close anatomic relationship between perivascular EAT and coronary arteries and the positive correlation between EAT and the presence of coronary atherosclerosis, several results point to EAT as a putative actor in CAD and/or T2DM [3, 10]. EAT thickness is an independent risk factor for CAD. EAT could thus be able to modulate heart and coronary artery pathophysiology, and mounting evidences point to EAT as a candidate player in pathophysiology of CAD. miRNAs act on multiple targets and complex pathogenesis, thus representing candidate regulators of adipocyte

differentiation, metabolic homeostasis, and inflammation. miRNAs have also been described as differentially modulated in adipose tissue during metabolic disease, thus being considered candidate biomarkers for metabolic disorders, CAD, T2DM, and putative targets for therapy [28]. We present evidence that a profile of miRNAs was dysregulated in EAT in CAD patients with T2DM compared to metabolic health patients, supporting the concept that miRNAs in EAT are involved in pathogenesis of CAD and/or T2DM. The current study identified a total of 11 differentially expressed miRNAs, and among them, hsa-miR-4687-3p drew specific attention for the largest targeted genes which were identified. Particularly, bioinformatics analysis revealed that insulin signaling pathway is the central way involved in the progression of metabolic disorders. Previous studies indicated that the circulating levels of miR-133a can be used as a predictor for diagnosing CAD, since increased miR-133a level may be used to predict both the presence and severity of coronary lesions in CAD patients [29]. Evidence from a recent study had showed that miR-370 was significantly increased in patients with T2DM and CAD and CAD only and patients with T2DM [23]. This was also concomitant with another study that showed the use of certain miRNA imprints including miR-370, in the screening of patients at risk for developing CAD [30]. Because of the highly metabolic paracrine and endocrine functions of EAT, it has been proposed to play a role in the pathogenesis of CVD by releasing proinflammatory and proatherogenic factors. EAT plays important roles in CAD, not only in its location, but also by its blood supply. EAT derives its blood supply from coronary circulation. There is a functional and anatomic relationship between EAT and muscular components of the heart as these components share the same coronary blood supply. The release of proinflammatory and proatherogenic factors into the circulation advancing CVD is more significantly linked to the progression of CAD [10]. It is the close anatomical relationship between EAT and the coronary arteries combined with its biologically active properties that participates in the pathogenesis of diabetic coronary atherosclerosis [31]. EAT-specific miRNAs are miR-196b-5p, miR-196a-5p (a promoter of brown adipogenesis), miR-18a-3p (a member of the miR-17/92 cluster that promotes adipocyte differentiation), and miR-10a-3p (an anti-inflammatory agent) which were analyzed by one article that also supported our study. In EAT of CAD patients, miR-135b-3p (a direct target of inflammatory pathways) was found upregulated, while miR-455-3p (a driver of during brown adipocyte differentiation), miR-193b-3p (promoting adiponectin secretion in human adipocytes), and Let-7a-3p and miR-127-3p (negative modulators of inflammatory pathways) were found downregulated [32, 33]. These findings are in accordance with our results that miRNAs are involved in the pathogenesis of metabolic disorders in EAT of CAD patients with T2DM. Overall, we were able to depict a novel miRNA signature of EAT in CAD patients with T2DM characterized by dysregulated miRNAs profile which are probably involved in pathogenesis of CAD. The results of the current study support the hypothesis that miRNA expression is deregulated in epicardial adipose tissue in patients suffering from T2DM and CAD disease.

We provided a comprehensive potential novel miRNAs expression signature of EAT in CAD patients with T2DM, and we showed that the targets of the miRNAs are necessarily associated with the pathogenesis of CAD and T2DM.

Disclosure

Yang Liu and Wenbo Fu should be considered co-first authors.

Competing Interests

The authors declare that they have no competing interests regarding the publication of this paper.

Authors' Contributions

Yang Liu and Wenbo Fu contributed equally to this work.

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