miR-146a in Cardiovascular Diseases and Sepsis: An Additional Burden in the Inflammatory Balance?

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Abstract

The new concept of thrombosis associated with an inflammatory process is called thromboinflammation. Indeed, both thrombosis and inflammation interplay one with the other in a feed forward manner amplifying the whole process. This pathological reaction in response to a wide variety of sterile or non-sterile stimuli eventually causes acute organ damage. In this context, neutrophils, mainly involved in eliminating pathogens as an early barrier to infection, form neutrophil extracellular traps (NETs) that are antimicrobial structures responsible of deleterious side effects such as thrombotic complications. Although NETosis mechanisms are being unraveled, there are still many regulatory elements that have to be discovered. Micro-ribonucleic acids (miRNAs) are important modulators of gene expression implicated in human pathophysiology almost two decades ago. Among the different miRNAs implicated in inflammation, miR-146a is of special interest because: (1) it regulates among others, Toll-like receptors/nuclear factor-κB axis which is of paramount importance in inflammatory processes, (2) it regulates the formation of NETs by modifying their aging phenotype, and (3) it has expression levels that may decrease among individuals up to 50%, controlled in part by the presence of several polymorphisms. In this article, we will review the main characteristics of miR-146a biology. In addition, we will detail how miR-146a is implicated in the development of two paradigmatic diseases in which thrombosis and inflammation interact, cardiovascular diseases and sepsis, and their association with the presence of miR-146a polymorphisms and the use of miR-146a as a marker of cardiovascular diseases and sepsis.

Keywords
- polymorphisms
- miRNA
- cardiovascular disease

Introduction

Thromboinflammation relies on the interplay between thrombosis and inflammation (through the activation of the hemostatic and coagulation system and the innate and adaptive immunity) and it has been recognized as a pathophysiological process in response to a wide variety of sterile and non-sterile stimuli causing in some cases acute organ damage.[1,2] Of special interest in this field are micro-ribonucleic acids (miRNAs), and among them, we here highlight miR-146a. This miRNA has been extensively studied given its prominent regulatory role in inflammatory and immune processes.[3,4] Additionally, genetic and epigenetic regulation

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of miR-146a expression in humans provide excellent models to evaluate the role of this miRNA in different pathologies.[5–7]

In this review, we will summarize the biology of miR-146a and its function in the interplay between inflammation and thrombosis, especially focusing in cardiovascular diseases (CVDs) and sepsis. These pathologies have been over the years separated in different categories. Interestingly, it has recently been proposed that they both share pathophysiological signaling pathways and common genetic variants.[8] Thus, both diseases share similar endpoints of inflammation, coagulation, and endothelial activation.[9,10]

Biosynthesis of miRNAs

It has been almost two decades since miRNAs were discovered as important regulators of gene expression in disease.[11] miRNA maturation is a complex process that starts in the nucleus where miRNA genes are transcribed by RNA polymerase II, giving rise to a product called pri-miRNA that is further processed into pre-miRNA by the microprocessor catalytic complex, composed by DROSHA and DGCR8. The resulting pre-miRNA molecule is a 3′overhang hairpin-like structure of approximately 60 nucleotides. The pre-miRNA is then exported to the cytoplasm by a RanGTP-dependent exportin (XPO5) allowing an additional processing step by DICER resulting in a new approximately 22 nucleotide mature double-strand miRNA.[12] At that point, a single-stranded miRNA is inserted in a functional macromolecular unit, containing among other the argonaute protein 2 (AGO2), recognizing its target messenger RNA (mRNA) by base pairing, mainly in the 3′ untranslated region, and allowing the inhibition of mRNA translation and/or the mRNA decay.[13] To date, 2,654 mature miRNAs (Mirbase, Release 22.1: October 2018) have been described in humans, that may repress the majority of the transcriptome, regulating different physiological and pathological processes.[14]

miR-146a Genetics and Regulation

The gene encoding mature miR-146a (*MIR146A*) is located on chromosome 5 (5q33.3) in humans and on chromosome 11 (B1.1) in mice. As it happens for the majority of miRNAs, miR-146a is highly conserved in mammalian (www.mirbase.org). The mature sequences were first assigned the name of the miRNA with or without an asterisk, that is, miR-146a (the predominant product) and miR-146a* (from the opposite arm of the precursor), but later (2011) a definitive nomenclature, miR-146a-5p (from the 5′ arm) and miR-146a-3p (from the 3′ arm), was established. In the case of miR-146a, the mature form that has been more extensively studied is miR-146a-5p. Since our studies and the references included in the review refer to this form, we used miR-146a throughout the text. Indeed, only a few studies have analyzed the role of miR-146a-3p and they were not included in the present review. Additionally, letter suffix denotes closely related mature sequences, in the case of miR-146a, there is a close miRNA, miR-146b (located in chromosome 10) that differs from miR-146a by two nucleotides located in the 3′ end, both miRNAs share the same seed region (located in the 5′ end) and most of the targets. The regulation of miR-146a expression is complex and may be performed at different levels as we will describe below. miR-146a transcription is executed by a promoter located 16 kb upstream of *MIR146A*.[15] Different putative binding sites for a series of transcription factors were characterized in this promoter (►**Fig. 1A**). Taganov et al demonstrated that miR-146a expression was mainly regulated by nuclear factor-κB (NF-κB) through Toll-like receptors (TLRs) such as TLR4 that is activated by lipopolysaccharide (LPS). In addition, other cytokines such as interleukin-1β (IL-1β) or tumor necrosis factor (TNF-α) also increased the levels of mature miR-146a.[15] Another study revealed that the transcription factor ETS-1 could potentially regulate the expression of miR-146a.[16] Indeed, an ETS-1 knockdown model provokes the inability to induce miR-146a expression in vitro.[17] On the opposite side, in vitro models showed that a c-Myc binding site, located in the promoter region, is able to repress the expression of miR-146a.[18] But other regulatory processes also take place at a genetic level. Different single-nucleotide polymorphisms (miR-SNPs) have been described to modulate miR-146a levels.[17] In this review, we will focus on three, rs2431697, rs2910164, and rs57095329, that have been extensively studied (►**Fig. 1A**). The first functional miR-SNP to be characterized was rs57095329 (MAF 0.26 in Asians and 0.025 in Europeans; https://www.ncbi.nlm.nih.gov/snp/).[17] This miR-SNP is located in the miR-146a promoter and the G allele decreases the binding affinity of ETS-1, reducing miR-146a levels by approximately 40%.[17] Another widely studied miR-SNP is rs2910164 (MAF 0.70 in Asians and 0.24 in Europeans; https://www.ncbi.nlm.nih.gov/snp/) that is located in the pre-miR-146a. The minor C allele causes mispairing within the hairpin affecting the efficiency of pri-miR-146a processing and probably the stability and/or efficiency of pre-miR-146a export to the cytoplasm[19] and reducing the levels by more than 40% in CC homozygous.[20] Rs2910164 has been associated with various diseases where inflammation is an important issue.[7,21,22] Finally, rs2431697 is another miR-SNP located approximately 30 kb upstream of pre-miR-146a. Löfgren et al[20] showed that the presence of the minor T allele reduces both pri-miR-146a levels as well as mature miR-146a levels (∼50%). The mechanism is not well defined and other miR-SNPs in linkage disequilibrium may be involved.[20] We will thoroughly develop the effects of rs2431697 together with rs2910164 in thrombosis in pathologies with inflammatory background in the next paragraphs.

Other factors such as long non-coding (lnc)-RNAs are newly described regulators of miR-146a. These molecules can modulate miR-146a expression preferentially by a sponging mechanism (►**Fig. 1B**). Thus, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) knockdown lead to reduction of phosphorylated inhibitor of NF-κB in a rat model of LPS-induced acute kidney injury[23] and suppressed inflammatory response by upregulating miR-146a in LPS-induced acute lung injury.[24] LncRNA X inactivate-specific transcript (XIST), in turn, by sponging miR-146a, diminished the mechanical pain threshold due to the upregulation of voltage-gated sodium channel 1.7 ($Na_v1.7$) in an animal pain model.[25] Finally,

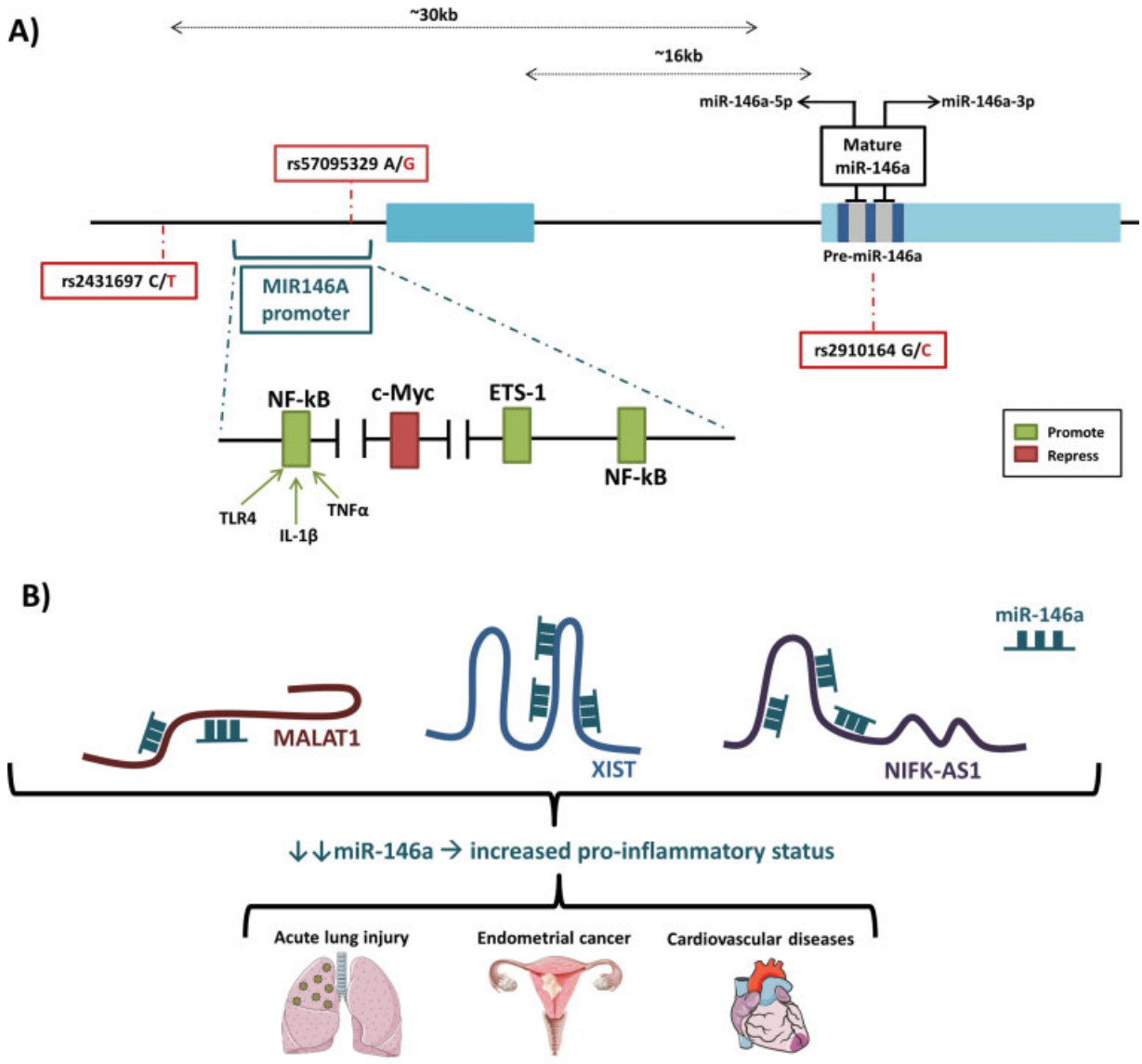

Fig. 1 miR-146a location and regulation. (**A**) miR-146a encoding gene is located in the human chromosome 5 (5q33.3). miR-146a transcription depends of a promoter located ~16 kb upstream of the *MIR146A* gene. Putative binding sites for transcription factors are shown as well as the location of the miR-single-nucleotide polymorphisms (SNPs) than can modulate miR-146a levels. (**B**) miR-146a may also be modulated by several long non-coding ribonucleic acids (Lnc-RNAs), generally by a sponging mechanism, this may lead to a higher inflammatory status that may be derived in the aggravation of certain pathologies.

in endometrial cancer, NIFK antisense RNA 1 (NIFK-AS1) inhibited the M2-like polarization of macrophages reducing the estrogen-induced proliferation, migration, and invasion of cancer cells by reducing miR-146a-5p levels through a sponging mechanism.[26]

Biological Functions of miR-146a in Immune Cells

miR-146a is a critical molecular brake of inflammation that regulates among other the TLR4/NF-κB pathway. Indeed, Taganov et al demonstrated in THP-1 cells that miR-146a directly modulates the expression of TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) that play an essential role in controlling the TLR4/NF-κB pathway[15] (►**Fig. 2**). A few in vitro studies have shown that miR-146a may also regulate the expression of TLR4, but in vivo studies are mandatory to further confirm this important regulation.[27–29] As mentioned above, LPS through TLR4/NF-κB promotes the expression of inflammatory cytokines such as IL-6, IL-8, IL-1β, or TNF-α and miR-146a controls overwhelmed cellular response to inflammatory signals through a negative feedback regulatory loop.[15,30,31] In 2011, Baltimore's laboratory published two studies using a deficient mouse model for miR-146a that gave many clues on the pathological role of miR-146a.[30,32] These mice react to LPS challenge with an important inflammatory response displaying high levels of IL-6 in serum, among other. Interestingly, aging also provokes them an increased inflammatory status. Indeed, the authors showed that aged *miR-146a*$^{-/-}$ mice developed a myeloproliferative phenotype and tumors in their secondary lymphoid organs.[30] These data suggest a role for miR-146a far beyond the

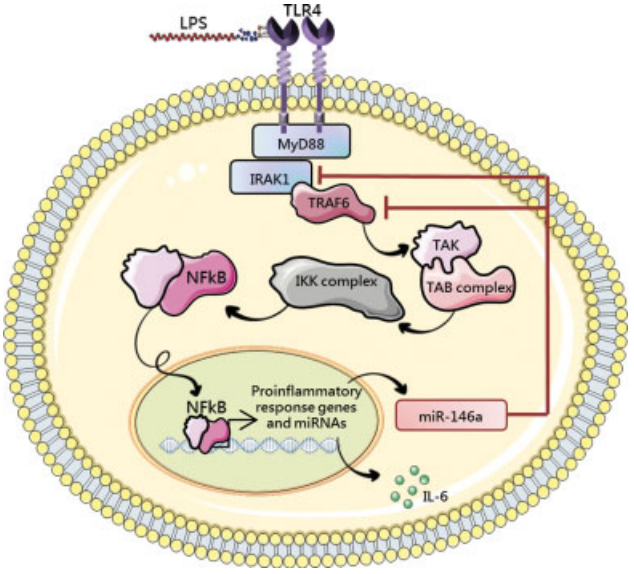

Fig. 2 Regulation of the Toll-like receptor (TLR) 4/nuclear factor-kB (NF-kB) axis by miR-146a. Binding of lipopolysaccharide (LPS) to TLR4 leads to the activation of NF-κB and its translocation to the nucleus. NF-κB induces the transcription of several proinflammatory genes (such as interleukin [IL]-6) and also promotes the transcription of pri-miR-146a that after a series of maturation processes gives rise to the mature miR-146a that will act through a negative feedback regulatory loop to essentially control the levels of TRAF6 and IRAK1, main players of the TLR4/NF-kB axis.

mere response to an endotoxin challenge since the deficiency of this miRNA constitutively affects NF-κB signaling.[30,32] In this line, our group recently demonstrated that rs2431697 TT genotype is an early predictor of myelofibrosis progression, independent of the JAK2V617F allele burden.[33] Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a critical role in myeloproliferative neoplasms pathogenesis by driving both the malignant clone and the inflammatory microenvironment. Thus, JAK2V617F allele burden is one of the main drivers of clonal expansion toward end-stage disease.[33] Our results showed that rs2431697 genotype increases Jak/Stat signaling, as we demonstrated in *miR-146a*$^{-/-}$ mice, probably due to the elevation of systemic IL-6 levels.[33,34]

One of the crucial elements implicated in thrombosis-linked inflammation is the neutrophil. These cells are mainly involved in eliminating pathogens and being an early barrier to infection.[35] In 2004, Brinkmann et al, in a landmark study, characterized a new mechanism, later termed NETosis, by which neutrophils are able to remove bacteria under certain stresses such as infection.[36] NETs are structures composed of nuclear chromatin and associated with nuclear histones as well as cytoplasmic and granular antimicrobial proteins.[37] Their association with CVDs, venous thrombosis, and autoimmune diseases has been largely documented.[37–39] We recently showed that miR-146a was involved in NET formation using a miR-146a$^{-/-}$ mouse model. Our results demonstrated that miR-146a deficiency promotes in vitro the intrinsic capacity of neutrophils to form NETs in response to phorbol 12-myristate 13-acetate.[40] On the other hand, sterile stresses such as atherosclerosis and non-sterile such as endotoxemia produced an increased in NETosis in *miR-146a*$^{-/-}$ mice versus wild-type (WT).[40] Although the precise mechanism is unknown, the lack of miR-146a provoked changes in neutrophils that display an aging phenotype characterized by the markers CD62L^{low} CD11b^{high} Cxcr4^{high}, and an overexpression of Tlr4 in the aged population.[41] miR-146a$^{-/-}$ neutrophils also showed a lower expression of Cxcr1, that has been associated with a proinflammatory phenotype. All these features together with an increased formation of reactive oxygen species observed in miR-146a$^{-/-}$ mice, by a yet to discover mechanism, may explain in part why miR-146a deficiency increases NETosis.[41]

In addition, miR-146a has a key role in macrophages polarization and tolerance, relevant processes in atherosclerosis development.[42] Importantly, miR-146a deficiency promoted M1 phenotype and its overexpression resulted in M2 macrophages by decreasing the expression of NOTCH receptor 1 (NOTCH1) an important regulator of macrophage differentiation and activation (►**Fig. 3A**).[43,44] This miRNA also inhibited the differentiation to M1 of hepatic macrophages by targeting signal transducer and activator of transcription 1 (*STAT1*) and consequently the interferon-γ signaling.[45]

Oxidized low-density lipoprotein (oxLDL), an important inducer of atherosclerosis, may also regulate miR-146a expression. OxLDL-stimulated macrophages presented a

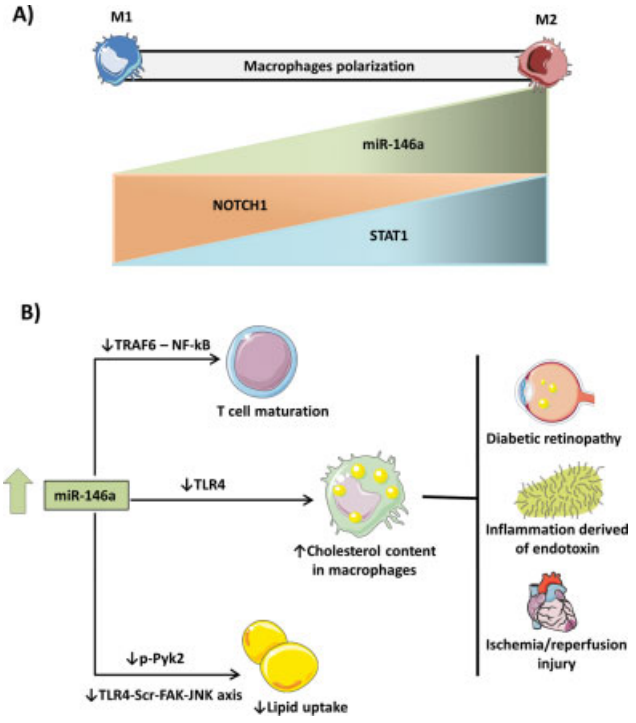

Fig. 3. Role of miR-146a on atherosclerotic inflammatory components. (**A**) miR-146a deficiency promotes macrophage polarization to M1 phenotype. Conversely, miR-146a overexpression promotes M2 phenotype by decreasing the expression of NOTCH1 and inhibited the differentiation to M1 by targeting STAT1. (**B**) miR-146a overexpression can block the TLR4-Src-FAK-JNK axis and inhibit the Pyk2 phosphorylation leading to the inhibition of lipid uptake. This overexpression can also decrease the nuclear factor-kB (NF-κB) activity through repressing TRAF6, resulting in T cells maturation. By provoking a lower TLR4 expression and higher intake of cholesterol by macrophages, the high levels of miR-146a may finally lead to diabetic retinopathy, inflammation derived of endotoxin, or ischemia/reperfusion injury, among other pathologies.

downregulation of miR-146a through lectin-type oxLDL receptor 1 (LOX-1).[46] In contrast, miR-146a overexpression provoked a reduction in TLR4 expression and higher cholesterol content in macrophages.[28] miR-146a may inhibit lipid uptake by blockade of TLR4–Src–FAK–JNK axis and the phosphorylation of protein tyrosine kinase 2 β (Pyk2) and paxillin, a signal transduction adaptor protein.[28] Accordingly, the expression of IL-6, IL-8, monocyte chemoattractant protein-1, and matrix metallopeptidase 9 decreased via miR-146a via TLR4-TRAF6/IRAK1-AKT.[46] Indeed, the direct repression of TLR4 by miR-146a has been extensively characterized in different pathologies such as diabetic retinopathy, inflammation derived of endotoxin, and ischemia/reperfusion (I/R) injury (►**Fig. 3B**).[29,47,48] On the other hand, miR-146a has also a role in adaptive immunity regulating T cell maturation and response against acute or chronic inflammation in a TRAF6-NF-κB-dependent manner.[49,50]

Relevance of miR-146a in the Pathophysiology Underlying Thromboinflammatory Diseases

Atherosclerosis

Atherosclerosis is the main underlying process driving to most cardiovascular pathologies including coronary artery disease (CAD), ischemic stroke (IS), or acute myocardial infarction (AMI).[51] Progressing atherosclerotic plaques may eventually rupture, thereby inducing intraluminal thrombosis leading to adverse cardiac events. Since inflammation is a key contributor to all stages of atherosclerosis and its fatal cardiovascular consequences, miR-146a was assumed to be a promising anti-inflammatory and atheroprotective agent. Nonetheless, apolipoprotein E (ApoE) controls inflammation by suppressing NF-κB signaling and protects from atherosclerosis and inflammatory diseases.[51] Li et al[52] reported that ApoE increased the expression of transcription factor PU.1 raising the levels of miR-146a in monocytes/macrophages and repressing the NF-κB signaling. Importantly, systemic intravascular delivery of miR-146a attenuated macrophage activation, atherosclerosis, and proinflammatory response in both, *ApoE* $^{-/-}$ and *Ldlr*$^{-/-}$ hyperlipidemia mouse models.[52] These findings established for the first time that enhancing miR-146a expression could antagonize atherogenesis. Intriguingly, we observed that lack of miR-146a exclusively in the hematopoietic compartment did not affect atherosclerotic plaque formation in *Ldlr*$^{-/-}$ mice at early and late stages of disease progression.[53] Similarly, Cheng et al[54] showed no differences in atherosclerotic burden in *Ldlr*$^{-/-}$ mice transplanted with *miR-146a*$^{-/-}$ bone marrow (BM) at early stages of disease despite having elevated levels of circulating proinflammatory cytokines. However, these authors found that *miR-146a*$^{-/-}$ mice receiving WT BM transplantation had enhanced endothelial cell activation and elevated atherosclerotic plaque burden compared with *Ldlr*$^{-/-}$ mice receiving WT BM, demonstrating the atheroprotective role of miR-146a in the endothelium.[54] In concordance with these results, it has been reported that endothelium-specific delivery of miR-146a-loaded E-selectin-targeting microparticles decreased plaque size and macrophage infiltration in *ApoE*$^{-/-}$ mice.[55] Thus, atheroprotection upon systemic miR-146a administration may therefore be caused by specific effects on vascular cells. In previous studies, the inhibition of NF-κB activation in endothelial cells reduced atherogenesis,[56] whereas inhibition of NF-κB activation in macrophages resulted in enhanced atherosclerosis in *Ldlr*$^{-/-}$ mice.[57] Additionally, miR-146a was enriched in extracellular vesicles (EVs) from mouse and human macrophages treated with an atherogenic stimulus (oxLDL), demonstrating an EV-mediated delivery of miR-146a that repressed target genes involved in cell migration and adhesion pathways in recipient cells.[58] Moreover, miR-146a levels were paradoxically elevated in plaques from atherosclerotic mice.[58] In clinical studies, miR-146a was also found to be overexpressed in valvular tissue from patients with atherosclerosis, suggesting an association of miR-146a with aortic valve stenosis.[59] Likewise, Raitoharju et al reported that miR-146a levels were elevated in plaque from aortic, carotid, and femoral atherosclerotic arteries versus non-atherosclerotic left internal thoracic arteries.[60] Interestingly, a study associated miR-146a levels with plaque stability in coronary stenotic lesions.[61] The authors found raised miR-146a levels in human peripheral blood mononuclear cells (PBMCs) from vulnerable plaque group versus those from stable plaque group.[61] Accordingly, miR-146a was also increased in PBMCs samples from CAD patients.[62] This apparent discrepancy with the animal studies showing that upregulation of miR-146a is atheroprotective might be explained by a compensatory upregulation of miR-146a in response to activation of NF-κB signaling in atherosclerosis, as part of a negative feedback loop. Overall, the role of miR-146a in atherosclerosis appears complex, and it is important to therapeutically focus on a specific cell type at a particular stage of atherogenesis.

Importantly, various association studies have indicated that *MIR146A* miR-SNPs (rs2431697 and rs2910164) play a role in atherosclerosis-related diseases (i.e., CAD and IS) development and progression. These studies have been performed in different populations and genetic make-up. Our group demonstrated that the T variant of rs2431697 (associated with low miR-146a levels) was predictor of adverse cardiovascular events, such as IS, in a cohort of 901 AF patients.[6] Subsequently, we evidenced that the TT genotype was associated with high inflammatory status and NET release, which could explain its prothrombotic effect.[40] In agreement with our findings, Wang et al[63] found that rs2431697 T carriers had an increased CAD risk in a Chinese population.

In contrast, the relationship between rs2910164 and CVD has been widely studied although the results are still inconclusive. Zhong et al[64] revealed that rs2910164 was associated with an increased risk of atherosclerotic cerebral infarction (ACI) in a Chinese cohort. Patients with reduced miR-146a expression in PBMCs exhibited an increased risk of ACI. Two additional studies evaluated the effect of rs2910164, finding that the G allele was associated with an increased risk of stroke or CAD.[65,66] Ramkaran et al described higher levels of miR-146a in PBMCs from young CAD patient carrying rs2910164 CC genotype.[67] These patients displayed significantly lower levels of IRAK1 and TRAF6, together with low

levels of NF-κB and C-reactive protein. Their observations implicated a protective function of CC genotype by increasing miR-146a levels and reducing inflammation in CAD patients.[67] However, other researchers have shown that the G allele of rs2910164 decreased the risk of CAD by downregulating the expression of miR-146a.[63,68,69] Hence, the inconsistency between results of several publications could be attributed to different ethnic groups and study designs. It is important to note that most of the studies were performed in Chinese populations and data gaps are evident in the Caucasian population. Clearly, further studies, including different geographical domains and larger sample sizes, are needed to globally understand the role of these miR-SNPs in CVD.

Myocardial Infarction

Several evidences directly link miR-146a and MI pathology. Notably, an altered expression of miR-146a has been found in autopsied heart tissue from MI patients compared with control hearts.[70] Indeed, MI patients with complications including ventricular rupture (VR) had upregulated tissue miR-146a levels versus those without VR.[70] The authors suggested that miR-146a increased in response to an intense inflammatory reaction that leads to the pathogenesis of VR after MI.[70] In addition, the role of miR-146a in heart diseases has been widely studied in animal models. However, it has not yet been determined whether its role is protective or harmful to the heart since miR-146a function varies depending on the heart disease model used. Wang et al[71] reported that miR-146a transfection into mouse hearts protected against myocardial I/R injury. miR-146a significantly decreased myocardial infarct size and attenuated myocardial apoptosis through the attenuation of NF-κB activation and inflammatory cytokine production by suppressing Irak1 and Traf6 expression.[71] Similarly, another recent study found that the injection of miR-146a-transfected human mesenchymal stem cells (hMSC-miR-146a) after myocardial I/R injury improved cardiac function in rats[72] by reducing the fibrotic area through the secretion of vascular endothelial growth factor.[72] A new finding indicated that exosomes derived from miR-146a-modified adipose stem cells play a key role in cardioprotection after MI by suppressing MI-induced apoptosis, inflammatory response, and fibrosis in an MI rat model.[73] Moreover, both in vivo and in vitro experiments found that miR-146a directly targeted early growth response factor 1, a well-known inducer of myocardial damage, and reverse MI or hypoxia-induced TLR4-NF-κB signal activation.[73] Another study in *miR-146a*$^{-/-}$ mice revealed that miR-146a deficiency increased infarct size and apoptosis after I/R injury through the upregulation of 19 apoptosis-related genes such as mediator complex subunit 1 (Med1).[74] On the other hand, tyrosine kinases inhibitors agents such as sunitinib (SNT), beyond their beneficial effects on cancer, have shown adverse effects on the cardiovascular system.[75] Interestingly, Shen et al showed a significant downregulation of miR-146a in the myocardium of SNT-treated mice.[75] Indeed, they demonstrated the protective effect of miR-146a upregulation on SNT-induced cardiac contractile dysfunction in vivo and in vitro by targeting cardiac phospholamban and ankyrin-2, both strongly involved in cardiac contractility.[75] Lastly, interesting data suggested that combination of miR-21 and miR-146a injection in mice (both with cytoprotective roles) had a greater protective effect against cardiac I/R-induced apoptosis compared with their individual effect.[76] Thus, Huang et al showed that this synergistic action was mediated by enhanced inhibition of apoptosis of cardiomyocytes by the miR-21/PTEN-AKT/p-p38 caspase-3 and miR-146a/TRAF6/p-p38 caspase-3 signal pathways.[76] Nevertheless, despite most studies attribute to miR-146a a cardioprotective role, others opposite them.[77–79] Particularly, it is worth mentioning the study by Oh et al that tested the effects of miR-146a modulation in transverse aortic constriction-induced heart failure (HF) models.[78] They demonstrated that overexpression of miR-146a attenuated cardiac contractile function by direct inhibition of the small ubiquitin-like modifier 1 (Sumo1) and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) expression, both previously associated with HF protection.

Therefore, further investigation is needed to clearly define the role of miR-146a during HF in particular and CVD in general, and to better define the cell-specific function of miR-146a under different pathological stresses.

Sepsis

Sepsis is the paradigm for inflammatory systemic diseases associating high morbidity and mortality in intensive care units.[80] Innate and adaptive immune systems' dysfunctions participate as drivers of this pathology in a network not fully known. Since activation of the coagulation cascade occurs in most patients with sepsis, the interplay between inflammation and coagulation has a crucial role in its pathophysiology.[81] Within hours after its initiation, the severe systemic inflammatory response shifts to an adaptive anti-inflammatory state caused in part by endotoxin tolerance that plays a key role in sepsis by limiting the negative consequences of an excessive inflammation and the endotoxin shock.[82] Several studies strongly support a crucial role for miR-146a in endotoxin tolerance by acting as a fine-tuning mechanism to prevent an overstimulation of the inflammatory response to persistent bacteria exposures.[47,83–86] In vitro experiments using human monocytic cells reported that miR-146a levels increased following LPS treatment (through TLR4) and negatively correlated with inflammatory cytokines as cells develop a status of LPS tolerance.[31] Importantly, tolerance induction required miR-146a upregulation and transfection of exogenous miR-146a prompted endotoxin tolerance, even in the absence of LPS priming.[31] Subsequent works confirmed these findings and showed that miR-146a was necessary for LPS-induced cross-tolerance to different TLR ligands.[31,87,88] Interestingly, miR-146a protection in endotoxin tolerance was also observed in morphine treatment, the main analgesic used in postoperative pain management, and a prevalent recreational drug with well-known adverse effects on the immune system.[85] Chronic morphine treatment mitigated endotoxin tolerance, resulting in persistent inflammation, septicemia, and septic shock by

downregulating LPS-induced miR-146a in macrophages.[85] Complementary studies by using various animal models have confirmed the critical role of miR-146a in the control of inflammation and organ dysfunction during bacteria or endotoxin-induced sepsis, involving macrophages as a major mechanism of innate immunity defense. In 2019, Funahashi et al demonstrated that miR-146a induction in splenic macrophages led to the attenuation of excessive inflammation, mortality rate, and severity of organ injury from polymicrobial sepsis induced by cecal ligation puncture (CLP) in mice.[89] Another recent work reported the protective role of miR-146a in LPS-induced organ damage and in the inflammatory response in mice by inhibiting the Notch1 signal in macrophages. This finding suggested miR-146a-Notch1-NF-κB axis as a potential target for the treatment and prevention of sepsis.[90] Concordantly, *miR-146a*$^{-/-}$ mice succumbed earlier than WT to septic shock induced by LPS.[30] Pan et al provided evidence supporting Jumonji domain-containing protein D3 (JMJD3), an histone lysine demethylase, as an epigenetic regulator of miR-146a transcription in an *Escherichia coli*-induced sepsis model.[91] The authors showed that inhibition of JMJD3, upregulated miR-146a transcription in peritoneal macrophages, protecting mice against early septic death.[91] Alternatively, Song et al found in a CLP-induced sepsis that miR-146a upregulated by IL-1β was selectively packaged into exosomes, transferred to recipient macrophages, where it regulated M1-M2 transition, and finally led to reduced inflammation and increased survival in septic mice.[92]

miR-146a dysregulation has also been described in sepsis in humans. With an interesting approach, Braza-Boïls et al have described that miR-146a was significantly downregulated in plasma after LPS treatment in an experimental human model of low-dose endotoxemia in volunteers.[93] These results suggest that LPS alone could also be inducing changes in miR-146a expression in humans. Furthermore, miR-146a dysregulation has also been associated with clinical manifestations of sepsis. The downregulation of miR-146a in PBMCs from septic patients have been correlated with elevated IL-6 and monocyte proliferation.[94] Indeed, IL-6 levels directly correlated with Sequential Organ Failure Assessment score in these patients.[94] In addition, a couple of reports showed indirect associations between miR-146a miR-SNPs, its targets and sepsis. Shao et al found for the first time a significant association between rs2910164 genotype (but not with rs57095329), miR-146a levels, and the susceptibility for sepsis,[80] although they could not demonstrate that miR-146a targets were regulated by this miR-SNP. Probably, clinical heterogeneity of these patients and their treatments masked the relation between miR-146a targets and the inflammatory status of septic patients.[80] Meanwhile, Han et al, reported that rs2910164 genotype conferred a worse outcome in those patients.[95]

On the other hand, cardiovascular complications are major consequences of sepsis/septic shock and are closely associated with increased morbimortality. Hence, Gao et al demonstrated that overexpression of miR-146a into mice myocardium subjected to CLP-induced sepsis protected them against cardiac dysfunction by markedly reducing the infiltration of macrophages and neutrophils into the myocardium and attenuating inflammatory response in both cardiomyocytes and macrophages via suppression of Nf-κB activity through Irak1 and Traf6 downregulation.[96] Moreover, a subsequent in vitro study reported that the overexpression of miR-146a mitigated the damage of cardiomyocytes induced by LPS in heart-derived H9C2 myocardial cells through negatively regulating NF-κB activation and inflammatory cytokine production via targeting the type I receptor protein tyrosine Erbb4.[97] In addition, Xie et al reported that miR-146a, through regulating Tlr-4/Nf-κB signaling pathway, improved inflammation and decreased myocardial injury markers in rats treated with LPS.[98] Thus, all these findings support that miR-146a could be a useful agent for protection against sepsis-induced cardiac dysfunction. In fact, we have demonstrated an association between miR-146a rs2431697 genotype and risk for cardiovascular events in CAP patients.[41] Thus, among 30 hospitalized patients with cardiovascular events, 29 carried the T allele (relative risk = 9.61, 95% confidence interval 1.28–72.15). Increased cardiovascular risk remained significant for T carriers 30 days after hospitalization.[41] Interestingly, our results pointed to NETosis as a functional way by which miR-146a levels lead to thrombosis in sepsis. Thus, among patients with the highest plasma levels of deoxyribonucleic acid/citrullinated histone H3 (citH3), those bearing T allele were threefold more frequent than CC. Furthermore, *miR146a*$^{-/-}$ mice injected with LPS presented higher citH3 and thrombin-antithrombin complex levels in plasma than WT and more severe lung injury. Based on these results miR-146a might have a role in immunothrombosis in septic patients.[41] The relationship between miR-146a levels and NETosis in sepsis evolution is an interesting field that we are exploring.

miR-146a as a Plasma Marker

Cardiovascular Diseases

After the discovery of miRNA stability in body fluids in 2008, a plethora of studies have evidenced the presence of a variety of circulating cell-free miRNAs.[99] The ability of these miRNAs to reflect physiological and pathophysiological conditions as well as their high stability in stored patient samples underlines the potential of these molecules to serve as biomarkers for several diseases.[100,101] Here, we focus on the potential of circulating miR-146a as a biomarker in thromboinflammatory disorders.

In accord with the protective role of miR-146a in atherosclerosis, Wagner et al reported that plasma levels of miR-146a were slightly downregulated in high-density lipoprotein (HDL) fraction from acute coronary syndrome (ACS) patients compared with healthy subjects.[102] However, this study has two major limitations: (1) the small sample size (10/group) and (2) that HDL-bound miR-146a fraction was less than 1% and did not correlate with total plasma levels of miR-146a. On the contrary, Oerlemans et al reported that serum miR-146a levels were significantly increased in those patients who developed an ACS, including those with negative high-sensitive cardiac troponin T (hs-cTnT) or < 3 hours of onset of chest pain. In

addition, circulating miR-146a levels discriminate non-ST elevation MI versus unstable angina.[103] These results were supported by a study showing that serum miR-146a levels were upregulated in CAD patients compared with controls.[104] In this study, although there were no differences in miR-146a levels between patients with stable versus unstable angina, miR-146a levels were significantly higher in MI versus stable angina patients. Interestingly, this study also showed that miR-146a was mainly associated with serum HDL.[104] In the same direction, Quan et al found a positive correlation between plasma miR-146a levels and the severity of coronary heart disease (CHD) measured by Gensini score. Interestingly, among CHD patients, those with subclinical hypothyroidism (SCH) exhibited the highest plasma miR-146a levels that positively correlated with thyroid-stimulating hormone levels, thus highlighting a potential predictive value of plasma miR-146a for CHD among individuals with SCH.[105] But the most enlightening study showed that plasma miR-146a levels were increased in MI patients compared with control subjects both, before and after percutaneous coronary intervention (PCI), showing a decrease of miR-146a levels after PCI.[106] They also found a significant positive correlation of miR-146a with other biomarkers such as N-terminal pro-brain natriuretic peptide and Hs-cTNT both, before and after PCI.[106] Interestingly, it has been proposed that miRNAs can be released into plasma during plaque rupture, thrombus formation, and myocardial I/R injury (necrosis and apoptosis), thus mirroring the levels found in the artery walls of origin.[106] Whether the increase in plasma miR-146a levels found in CAD/CHD/AMI patients in those studies may reflect a miR-146a-driven pathogenic or, on the contrary, a compensatory mechanism, has yet to be elucidated.

The alteration in circulating miR-146a levels has also been reported in other cardiovascular clinical entities. Halkein et al showed that levels of exosomal miR-146a were significantly higher in plasma from patients with acute peripartum/postpartum cardiomyopathy than in healthy postpartum controls and patients with dilated cardiomyopathy.[107] Kin et al examined tissue and plasma miRNAs specifically associated with atherosclerotic abdominal aortic aneurysm (AAA), and found that miR-146a was significantly upregulated in AAA tissue compared with normal aortic wall tissue, while it was significantly downregulated in the plasma of AAA patients compared with the plasma from healthy controls.[108]

Sepsis

Several studies have remarked the role of miR-146a as a promising prognostic and diagnostic biomarker for sepsis.[109] Wang et al reported that serum level of miR-146a was significantly reduced in patients suffering from sepsis compared with nonseptic systemic inflammatory response syndrome (SIRS) patients and healthy individuals, suggesting that miR-146a could distinguish sepsis from SIRS caused by other noninfectious diseases.[110] Subsequently, results from another study also showed higher serum miR-146a levels in nonsepsis SIRS patients compared with sepsis patients,[111] suggesting that miR-146a may be an optimal diagnostic tools for sepsis. Caserta et al confirmed the decrease of miR-146a in sepsis compared with noninfective SIRS.[112] Recently, Chen et al showed that high levels of miR-146a were associated with higher sepsis risk, disease severity, and systemic inflammation.[113]

Conclusion and Future Perspectives

Interaction between thrombosis and inflammation is a central feature of several highly prevalent pathologies. Thus, it is essential to understand the mechanisms and to discover new elements leading to thromboinflammatory processes. This is a key to develop new and effective therapeutic tools to fight thrombosis in several diseases. In this context, current evidences strongly support that miR-146a plays a relevant role in arterial thrombosis in diseases with an important inflammatory background. miR-146a is ubiquitously expressed and it exerts several effects in different cell types ranging from endothelial cells and macrophages in atherosclerosis to leukocytes in autoimmune diseases (►**Fig. 4**; ►**Table 1**). But many questions remain unanswered on the molecular mechanisms and processes that are controlled by miR-146a. Importantly, miR-146a is involved in NET formation although the precise mechanism is unknown.[40,41] Whether miR-146a affect other cells such as macrophages[114] or platelets[115] or processes that may affect NETosis have to be further searched. In addition, the discovery of new miR-146a targets with a role in thrombosis and inflammation may be determinant and approaches using genomics and transcriptomics in the adequate samples from the adequate patients or animal model may help in this endeavor. In this sense, another vital point is to know if miR-SNPs affecting miR-146a levels also produce neutrophil phenotypic changes as those observed in *miR-146a*$^{-/-}$ mice and would explain the association between the presence of these genetic alterations and thrombosis in patients with thromboinflammatory diseases such as those described in the present review.

Another interesting aspect is the use of miR-146a as a biomarker of a thromboinflammatory condition. In this case, there is still work to do concerning the standardization of protocols, and this is extensive to any miRNA. There are many limitations in the studies related with the use of miRNAs as markers of diseases and the best way of extraction and quantification from plasma, serum, other fluids, or EVs. Inconsistencies and variations in results between studies can mostly be attributed to preanalytical variation arising from different protocols as well as different normalization strategies. The use of plasma or serum, the purification technique, batch effects, the anticoagulant used, leukocyte and platelet contamination, hemolysis, sample storage time, and quantification technique are important factors that influence miRNA plasma level variability observed between studies.[116–119] In addition, to obtain enough statistical power, cohorts have to be well calculated to obtain consistent and reproducible results. How and when the samples are obtained also accounts for the large variability observed between studies. For example, drug treatment in CVD or in sepsis can modify miRNA expression levels and may confound the results as shown in several works from Mayr's laboratory among others.[93,116,120] Thus, given the potential

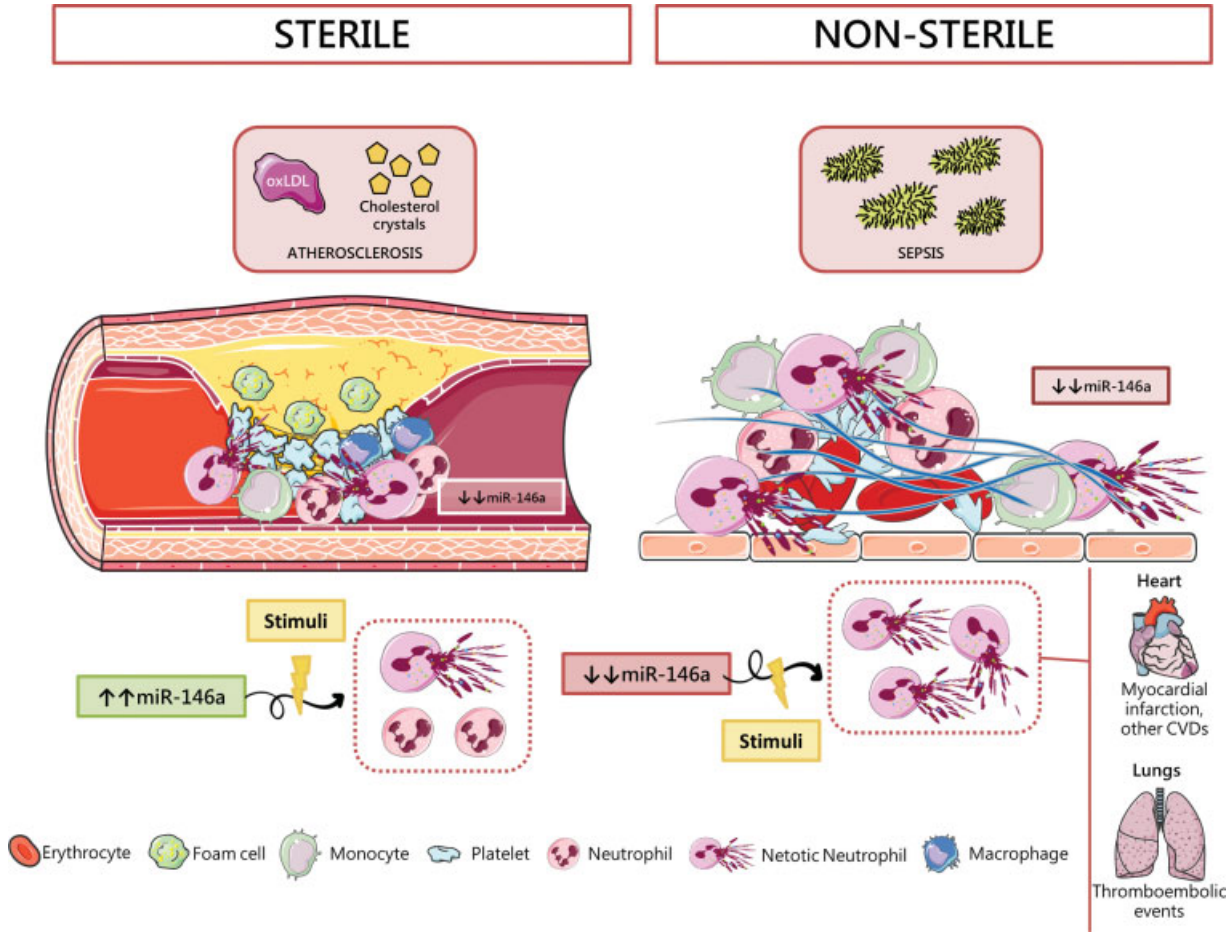

Fig. 4 miR-146a plays a relevant role in thromboinflammation in sterile and nonsterile conditions. The presence of miR-single-nucleotide polymorphisms (SNPs) such as rs2431697 and rs2910164 promote a phenotype with low levels of miR-146a. Upon sterile or nonsterile stimuli, neutrophils expressing the minor alleles would be more prone to NETosis leading to a higher thromboinflammatory process.

Table 1 List of genes targeted by miR-146a

Target gene	Functions	Reference(s)
IRAK1	Essential role in controlling the TLR4/NF-kB pathway	15
IRAK2	Component of the IL-1R signaling complex, promotes NF-kB signaling	123
TRAF6	Promotes NF-kB signaling	15
TLR4	Production of inflammatory cytokines via NF-kB	28
STAT1	Crucial role in IFN-γ signaling	45
NOTCH1	Regulator of macrophage differentiation and activation	43
NOTCH2	Development of marginal zone B cells	124
CXCR4	Chemokine receptor, involved in calcium mobilization, integrin-mediated adhesion, gene transcription, and proliferation	125
NUMB	Negatively regulated NOTCH signaling	124
SOD2	Radical scavenger, essential for balancing the intracellular ROS	126
EGR1	Promotes TLR4-NF-kB signal activation induced by hypoxia	73
CARD10	Specifically required for GPCR-induced NF-κB activation	127
COPS8	Controls NF-kB activation in activated T cells	127
IL-6, IL-8, CCL5	Chemokines for acute inflammatory responses	128
COX2	Prostanoid biosynthesis, involved in many age-related diseases	129

Abbreviations: GPCR, G-protein-coupled receptor; IFN-γ: interferon γ; ΙΛ, ιντερλευκιν; NF-κB, nuclear factor-kB; TLR, Toll-like receptor.

of this technique to diagnose or predict CVD an effort in standardization is necessary to accelerate its use in the future. However, there are already some miRNA panels that are commercially available for the diagnosis of certain pathologies and additional effort must be done to include new applications.[121]

The use of miR-146a as a therapeutic tool is also an exciting future application. Several studies in animal models have shown that miR-146a replacement therapy favors an anti-inflammatory status that could be beneficial in thromboinflammatory diseases such as CVD,[122] yet no clinical trials have started with miR-146a. Indeed, miRNA therapeutics employing miRNA mimics or antagomirs are currently under clinical trials in different diseases such as cancer, hepatitis C, or HF.[122] Thus, additional studies on potential off-target effects and efficient delivery methods are needed to allow envisioning miR-146a as an effective therapeutic drug against thromboinflammatory diseases.

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Conflict of Interest
None declared.

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