Section Editors: Martin Dichgans, MD, and Daniel Woo, MD, MS

Role of Non-Coding RNAs in Stroke

Steffen Tiedt, MD, PhD; Martin Dichgans, MD

Over the past decade, non-coding RNAs (ncRNAs) have increasingly been recognized as key regulators of cellular function in health and disease. ncRNAs have been studied both in the context of specific stroke etiologies, such as atherosclerosis, and in the context of stroke pathophysiology, such as excitotoxicity. Moreover, their presence in the circulation has triggered the idea that ncRNAs might serve as biomarkers for specific disease states and thus guide clinical decision-making. Accordingly, circulating ncRNAs have been the subject of multiple studies examining their potential clinical utility for risk prediction, diagnosis, and prediction of stroke outcome.

The abundancy of the ncRNA class is illustrated by the fact that, whereas about 3 quarters of the human genome are transcribed, <3 % are ultimately translated into proteins.[1] Also, the relative amount of DNA that is transcribed into ncRNA shows higher correlation with species complexity than the number of protein-coding genes,[2] thus pointing to the functional relevance of ncRNAs. Beyond housekeeping ncRNAs, such as ribosomal and transfer RNA, this has mostly been attributed to the myriad of non-coding regulatory RNA classes. By convention, ncRNAs are divided into long non-coding RNAs (lncRNAs) ≥200 nucleotides in length and small non-coding RNAs (<200 nucleotides), which include microRNAs (miRNAs), PIWI (P-element-induced wimpy testis-interacting) RNAs, and endogenous short interfering RNAs. Here, we summarize the emerging evidence for a regulatory role of miRNAs and lncRNAs in atherosclerosis and stroke pathophysiology. We further review current literature on their use as blood-based biomarkers and discuss opportunities and challenges for future studies aiming at translation into clinical application in diagnostics and therapy.

ncRNAs: Biogenesis and Modes of Action

MicroRNAs

miRNAs encompass a class of >2000 small ncRNAs with a length of ≈22 nucleotides. Their biogenesis and function have been extensively reviewed[3,4] and are summarized in Figure 1A. In short, depending on the developmental stage and cell type miRNA genes are transcribed into a primary hairpin miRNA. The Microprocessor complex, consisting of Drosha and DGCR8 (DiGeorge syndrome chromosomal region 8), cleaves the primary miRNA into a precursor miRNA. After nuclear export, the precursor miRNA is further processed by Dicer to generate a small RNA duplex. The functional guide strand of this duplex represents the mature miRNA and is loaded onto an Argonaute protein to form the RNA-induced silencing complex. Conformational rearrangements within the RNA-induced silencing complex allow the 6 to 8 nucleotides long miRNA seed region to scan for target messenger RNAs (mRNA; Figure 1A).[3] Binding of complementary target mRNAs evokes partial post-transcriptional silencing through mRNA destabilization and translational repression, thus leading to optimized or insufficient protein levels. Importantly, most miRNAs influence the expression of >1 target gene, which in turn may be regulated by >1 miRNA. Hence, phenotypic consequences may result from (I) 1 miRNA repressing 1 target, (II) 1 miRNA repressing multiple targets, or (III) multiple miRNAs repressing the same or even different targets cumulating in the same phenotype.[4] As such, miRNAs orchestrate cellular responses to pathophysiological stimuli. They are also present in blood and are protected from degradation by inclusion in extracellular vesicles like exosomes, microvesicles, or apoptotic bodies, or by association with RNA-binding proteins or lipoprotein complexes (Figure 1A). miRNAs can further be actively secreted from cells, which in light of the role of exosomes and microvesicles in paracrine signaling, implies a potential function of miRNAs in intercellular communication.[5]

Long noncoding RNAs

More than 58 000 transcripts represent the class of lncRNAs, some of them reaching several kilobases in length. They exhibit some coding function[6] but, like miRNAs, mostly have regulatory function, thereby adding another layer to cellular regulatory networks. Depending on their genomic relation to protein-coding genes lncRNAs are classified as sense, antisense, intergenic, intronic, and bidirectional.[7] Their dynamic secondary and tertiary structures and ensuing changes in binding sites allow them to interchangeably interact with DNA, other RNAs, and proteins. According to their molecular mechanism, lncRNAs can further be

Received July 12, 2018; final revision received September 17, 2018; accepted September 25, 2018.

From the Institute for Stroke and Dementia Research, University Hospital, Ludwig-Maximilians-Universität Munich, Germany (S.T., M.D.); and Munich Cluster for Systems Neurology (SyNergy), Munich, Germany (S.T., M.D.).

The Data Supplement is available at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.118.021010.

Correspondence to Steffen Tiedt, MD, PhD, Institute for Stroke and Dementia Research, Klinikum der Universität München, Ludwig-Maximilians-Universität LMU, Feodor-Lynen-Straße 17, Munich 81377, Germany. Email steffen.tiedt@med.uni-muenchen.de

(*Stroke*. 2018;49:3098-3106. DOI: 10.1161/STROKEAHA.118.021010.)

© 2018 American Heart Association, Inc.

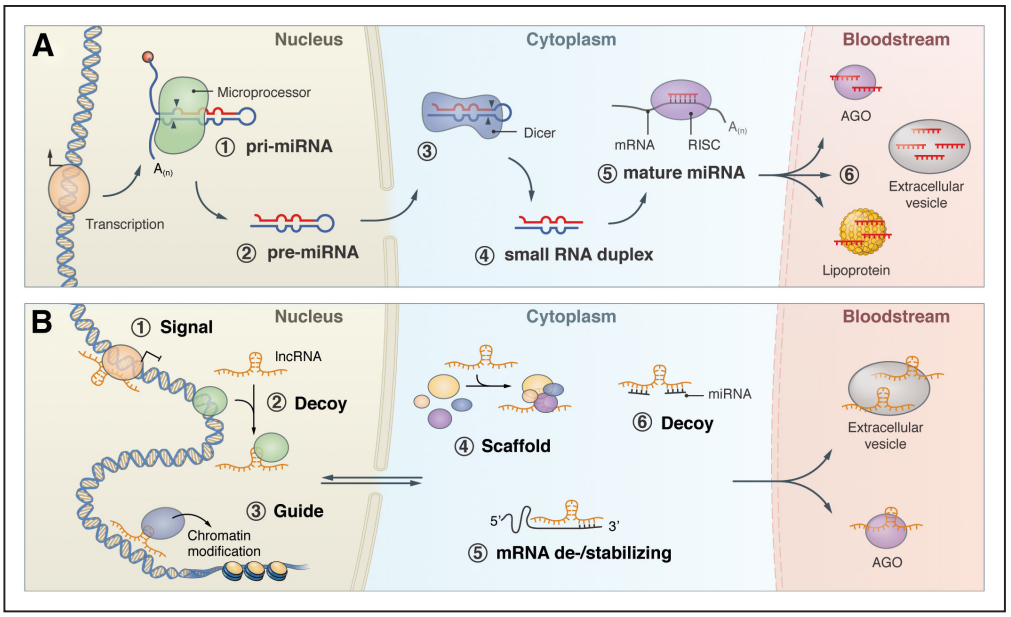


Figure 1. Biogenesis of microRNAs (miRNAs) and functions of long non-coding RNAs (lncRNAs). **A**, miRNA biogenesis is initiated in the nucleus (1–2) and completed in the cytoplasm (3–5). miRNAs escape degradation in blood by transportation in vesicles or by proteins (6). **B**, lncRNA functions can be categorized by molecular mechanism (1–4), cellular compartmentalization (1–3 vs 4–6) and by binding partners: DNA (3), RNA (5, 6), or proteins (1, 2, 4). Image by Dr Johannes Richers. AGO indicates Argonaute protein; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; and RISC, RNA-induced silencing complex.

organized within 4 not mutually exclusive classes: (1) signal lncRNAs, for example binding the chromatin remodeling and transcriptional machinery to silence transcription, (2) decoy lncRNAs, which bind and thereby expel proteins and miRNAs from their targets, (3) guide lncRNAs, for example chaperoning ribonucleoproteins to chromatin targets, and (4) scaffold lncRNAs, which bind distinct proteins to build complexes (Figure 1B). Depending on their cellular localization, lncRNAs may affect gene expression by binding the chromatin remodeling and transcriptional machinery in the nucleus or influence mRNA stabilization, translation, and decay in the cytoplasm (Figure 1B).[7] Yet, owing to their more recent discovery, knowledge about the functional role of single lncRNAs is still limited when compared with miRNAs. Similar to miRNAs, lncRNAs have also been found to circulate in the blood where they are bound to protein complexes or included in vesicles (Figure 1B).[5]

ncRNAs in Stroke Pathogenesis

The tissue- and developmental stage-dependent expression of ncRNAs indicates a high functional relevance in physiological and disease processes. Several observations suggest a causal role of ncRNAs in stroke. First, genome-wide association studies have identified a susceptibility locus for large-vessel stroke on chromosome 9p21 harboring the lncRNA *ANRIL* (antisense ncRNA in the INK4 locus).[8,9] Second, an analysis of 2763 participants from the population-based prospective Framingham Heart Study found circulating miR-656-3p and miR-941 to be associated with incident stroke.[10] Third, a highly penetrant mutation in a miR-29 binding site in the 3′ untranslated region of *COL4A1* causes pontine autosomal dominant microangiopathy with leukoencephalopathy (PADMAL).[11] In accord with these findings, experimental studies suggest a role of ncRNAs in key mechanisms of stroke pathogenesis, including atherosclerosis[12] and atrial fibrillation.[13] In the following, we will discuss ncRNAs with potential relevance to stroke prevention by influencing atherosclerosis (Table 1; Figure 2).

Atherosclerosis evolves across distinct stages including early dyslipidemia- and shear stress-mediated endothelial cell dysfunction, adhesion of leukocytes to endothelial cells, invasion of leukocytes into the intima, migration, and proliferation of smooth muscle cells (SMC), development of a fibrous cap, and eventually plaque rupture (Figure 2).[33] ncRNAs have been implicated in multiple aspects of atherogenesis. miR-33 has been shown to promote early atherogenesis by regulating the biosynthesis of HDL (high-density lipoprotein) and the reverse cholesterol transport pathway. miR-33a and miR-33b are located within introns of the genes encoding the sterol-regulatory element-binding factors and reduce circulating HDL levels by targeting the ABC (ATP-binding cassette) transporter *ABCA1*.[20] Inhibition of miR-33 in both mice and monkeys increased plasma HDL levels[20,21] and reduced plasma levels of VLDL (very-low-density lipoprotein)-associated triglycerides.[21] Moreover, miR-33 antagonism promoted the accumulation of regulatory T cells and anti-inflammatory macrophages within plaques, thereby leading to reduced plaque size in atherosclerosis-prone mice.[34]

Shear stress at predilection sites is another important factor aggravating endothelial dysfunction. Shear stress induces downregulation of miR-126-5p, the passenger strand of miR-126, in endothelial cells, and intravenous application of nanoparticle-carried miR-126-5p to atherosclerosis-prone mice for 4 weeks reduced aortic root lesion size by ≈75%,[24] thus highlighting the potential of miRNA-based interventions for atheroprotection.

After endothelial activation, inflammation of the arterial wall provokes the migration and proliferation of SMCs—a

Table 1. Key ncRNAs Regulating Atherosclerosis

ncRNA	Mechanism
ANRIL	Linked to large-vessel stroke by multiple GWAS,[8] *ANRIL* isoforms exert different functions: a circular *ANRIL* isoform inhibits proliferation of SMCs and macrophages,[9] whereas linear *ANRIL* transcripts are atheroprogressive.[14]
lincRNA-p21	*lincRNA-p21* confers atheroprotection by repressing proliferation and inducing apoptosis of SMCs and macrophages. Expression levels were reduced in atherosclerotic plaques in atherosclerosis-prone mice.[15]
MeXis	The macrophage-expressed LXR-induced sequence (*MeXis*) guides the transcriptional coactivator DDX17 to amplify *ABCA1* expression, thereby regulating cholesterol efflux and mediating atheroprotection.[16]
miR-21	miR-21 enhances proliferation of SMCs[17] and cholesterol efflux from macrophages.[18] Its ablation accelerated atherosclerosis including plaque necrosis despite decreasing SMC proliferation. Local delivery rescued the vulnerable plaque phenotype.[19]
miR-33a/b	Intronically located within the *SREBF* genes, miR-33 has strongly been linked to the biosynthesis of HDL and the reverse cholesterol transport pathway. Its inhibition increased HDL levels and decreased VLDL levels.[20,21]
miR-92a	The expression levels of this mechanosensitive miRNA are reduced in ECs on atheroprotective laminar flow patterns, thereby derepressing expression of the transcription factor KLF2 to maintain endothelial homeostasis.[22]
miR-122	This liver-enriched miRNA affects the expression of multiple genes in cholesterol biosynthesis.[23] It was also the target of the pioneering use of chemically modified oligonucleotides in vivo.
miR-126-5p	Shear stress reduces the expression of miR-126-5p in ECs, thereby diminishing the proliferative reserve of ECs. Long-term intravenous delivery of miR-126-5p reduced atherosclerotic plaque burden.[24]
miR-128	Proximally located to SNPs associated with abnormal blood lipids, miR-128 targets *LDLR* and *ABCA1*. Long-term inhibition decreased circulating total cholesterol levels by 35%.[25]
miR-143/-145	Shuttling between ECs and neighboring SMCs, miR-143 and miR-145 cooperate to maintain vessel wall function.[26] By targeting KLF4 and KLF5 and enhancing myocardin expression, they repress proliferation of SMCs.[27]
miR-146a	Expression of this cytokine-responsive miRNA in ECs is upregulated by proinflammatory cytokines. By targeting the NF-κB pathway, *HuR*, and the MAP kinase pathway, miR-146a blunts inflammation.[28]
miR-148a	In addition to its proximity to SNPs associated with abnormal blood lipids (like miR-128), miR-148a was also identified in a screen for miRNAs to regulate *LDLR* expression. Long-term inhibition elevated plasma HDL-C and decreased LDL-C.[25,29]
miR-181b	miR-181b represents another cytokine-responsive miRNA, whose expression in ECs is reduced in the course of atherosclerosis. By targeting the NF-κB pathway, miR-181b reduces leukocyte recruitment.[30]
miR-210	Unstable carotid plaques show decreased levels of miR-210. The delivery of miR-210 improved plaque stability by targeting *APC*, thereby affecting Wnt signaling and regulating SMC survival.[31]
miR-223	miR-223 regulates the expression of many genes involved in lipoprotein homeostasis, such as *SCARB1*, *HMGCR*, and *SP3*. Genetic ablation of miR-223 in mice resulted in increased HDL-C levels.[32]

ABCA1 indicates ATP-binding cassette transporter 1; APC, adenomatous polyposis coli; EC, endothelial cell; GWAS, genome-wide association study; HDL-C, high-density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HuR, human antigen R; KLF2, Krüppel-like factor 2; LDL-C, low-density lipoprotein cholesterol; LDLR, LDL Receptor; LXR, liver X receptor; MAP, mitogen-activated protein; miRNA, microRNA; ncRNA, non-coding RNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SCARB1, scavenger receptor class B type 1; SMC, smooth muscle cell; SNP, single-nucleotide polymorphism; VLDL, very-low-density lipoprotein; and Wnt, wingless-related integration site.

process that is regulated by multiple ncRNAs. First, a circular *ANRIL* isoform inhibits proliferation and induces apoptosis of both SMCs and macrophages by negatively regulating ribosomal RNA maturation, thereby conferring atheroprotection.[9] Interestingly, linear *ANRIL* transcripts were found to exert atheroprogressive effects by trans-regulation of related gene networks.[14] Second, the miR-143/miR-145 cluster promotes differentiation and represses proliferation of SMCs by reducing KLF (Krüppel-like factor) 4 and KLF5 and increasing myocardin expression.[27] miR-145 overexpression resulted in reduced plaque size and enhanced plaque stability.[35] Notably, miR-143 and miR-145 have been shown to shuttle between endothelial cells and neighboring SMCs using extracellular vesicles and tunneling nanotubes,[26] and thereby display synergistic atheroprotective effects in different cell types. Third, miR-21 enhances proliferation of SMCs, and its expression levels were elevated after in-stent restenosis.[17] Both genetic ablation of miR-21[17] and local delivery of anti-miR-21 by coated stents was protective by effectively reducing in-stent restenosis.[36] However, ablating miR-21 in macrophages accelerated atherosclerosis, including plaque necrosis by lowering cholesterol efflux and enhancing foam cell formation.[18] This may also explain why miR-21-deficient mice developed higher atherosclerotic burden, despite lacking SMC proliferation. Local delivery of miR-21 using ultrasound-targeted microbubbles reversed the vulnerable carotid plaque phenotype.[19] Hence, local delivery of miRNA-targeted drugs to overcome undesirable actions in other cell types might prove valuable for translating experimental findings to patients.

ncRNAs in Stroke Pathophysiology

Upon ischemia, brain cells undergo rapid changes in gene expression. Accordingly, both miRNAs[37] and lncRNAs[38] have been demonstrated to exert early expression changes in brain tissue after experimental ischemia. Both specific miRNAs and lncRNAs have been shown to regulate pathophysiological

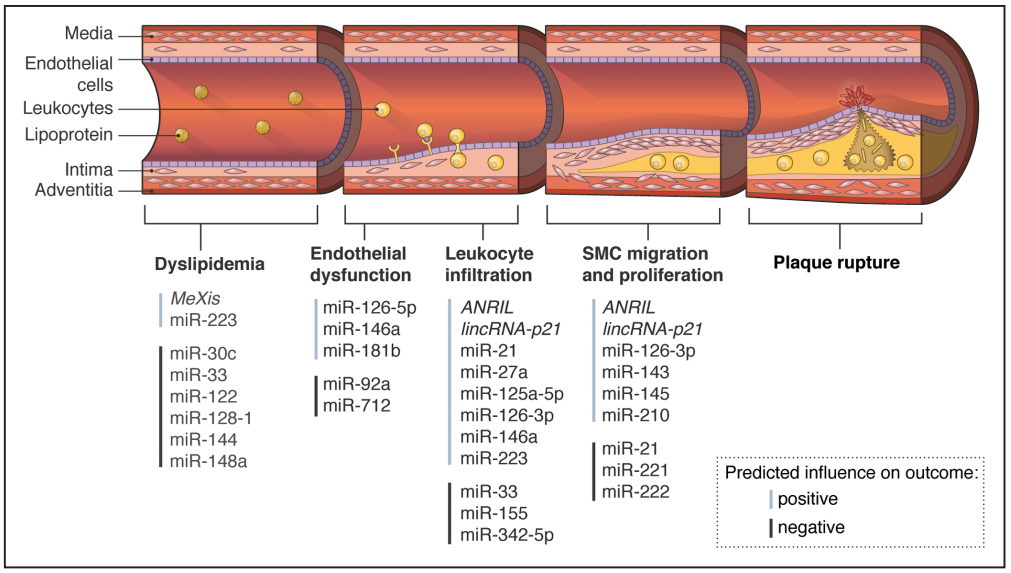


Figure 2. Non-coding RNAs (ncRNAs) regulating atherogenesis. Both specific long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) have been found to control distinct stages of atherosclerosis. Image by Dr Johannes Richers. SMC indicates smooth muscle cell.

processes after stroke, such as excitotoxicity, programmed cell death, inflammation, and blood-brain barrier breakdown (Figure 3), and, therefore, represent potential therapeutic targets in the acute and subacute phase of stroke.

In keeping with their capacity to fine-tune gene expression in regulatory networks, different ncRNAs have been shown to converge on the same pathophysiological mechanisms after stroke. For instance, >30 ncRNAs were reported to control programmed cell death after stroke (Figure 3). Conversely, many ncRNAs have been linked to more than a single process. A good example is miR-124, one of the most highly expressed miRNAs in the brain. miR-124 has been shown to regulate gene expression relevant for excitotoxicity, programmed cell death, inflammation, blood-brain barrier breakdown, angiogenesis, and neurogenesis after stroke (Figure 3). Accordingly, miR-124 has been shown to regulate >100 genes when transfected into HeLa cells,[39] which usually do not express miR-124. Yet, studies examining the effects of miR-124 poststroke have focused on single targets, such as the genes encoding iASPP[40] (an inhibitory member of the ASPP [apoptosis-stimulating proteins of p53] family) and Usp14[41] (a deubiquitinating enzyme) and the effects of altered miR-124 levels on stroke outcome are still debated.[40,41] To fully understand the effects of miR-124 on gene expression poststroke and to elucidate the potential of miR-124-targeted therapeutics for application in human stroke, future studies will need to (1) determine the kinetics of miR-124 expression in different cell types and brain regions poststroke, (2) explore miR-124-mediated changes in gene expression poststroke, for example, by untargeted proteomic analysis, (3) characterize the cellular uptake of therapeutics, their effects on miR-124 expression, and target repression in different cell types, and (4) determine their effects on the various mechanisms of stroke pathophysiology that have been linked to miR-124.

An example for the convergence of different ncRNAs on a specific pathophysiological mechanism is excitotoxicity, one of the earliest events after stroke (Figure 3). Aside from the effects of miR-124 on excitotoxicity, investigators found miR-223, a ubiquitously expressed miRNA, to decrease NMDA (*N*-Methyl-D-aspartic acid)-mediated calcium influx. miR-223 limits excitotoxicity by targeting the glutamate receptor subunit genes *GluR2* and *NR2B* and reduced lesion volume in a transient global ischemia model.[42] Another study found miR-1000 in *Drosophila* and its mammalian homolog miR-137 in the mouse to be neuroprotective by repressing the expression of the vesicular glutamate transporter.[43] However, these findings await functional confirmation in experimental stroke models.

ncRNAs have further been implicated in programmed cell death poststroke. Yan et al[44] even identified a functional link between a miRNA and the lncRNA *MEG3*. Specifically, *MEG3* was found to function as a sponge for miR-21 titrating it away from the programmed cell death 4 (*PDCD4*) mRNA. *MEG3* was elevated in brain tissue after transient focal ischemia, and its inhibition was associated with elevated levels of miR-21, decreased levels of *PDCD4* and decreased infarct volume.[44]

miRNAs have further been linked to blood-brain barrier breakdown and microvascular integrity poststroke (Figure 3). Caballero-Garrido et al[45] provided a rare example of an intravenously administered miRNA-targeted therapeutic that reduced infarct volume when administered after experimental stroke. They identified inhibition of miR-155 as a promising approach to improve microvascular integrity, potentially mediated by elevated levels of RHEB (Ras homolog enriched in brain) and stabilization of ZO-1 (tight junction protein-1).[45] The impact of miRNAs on microvascular integrity might also apply to hemorrhagic stroke: Xi et al[46] demonstrated a beneficial effect of miR-126-3p on blood-brain barrier permeability, edema formation, neuronal cell death, and functional outcome when administered after experimental intracerebral hemorrhage.

An unconventional way of signaling has been proposed for let-7 implicating a potential role in neurodegeneration after stroke: upon injury, cultured neurons released let-7, which in turn induced cell death via TLR (Toll-like receptor) 7 signaling when added to neurons in vitro and in vivo.[47] However,

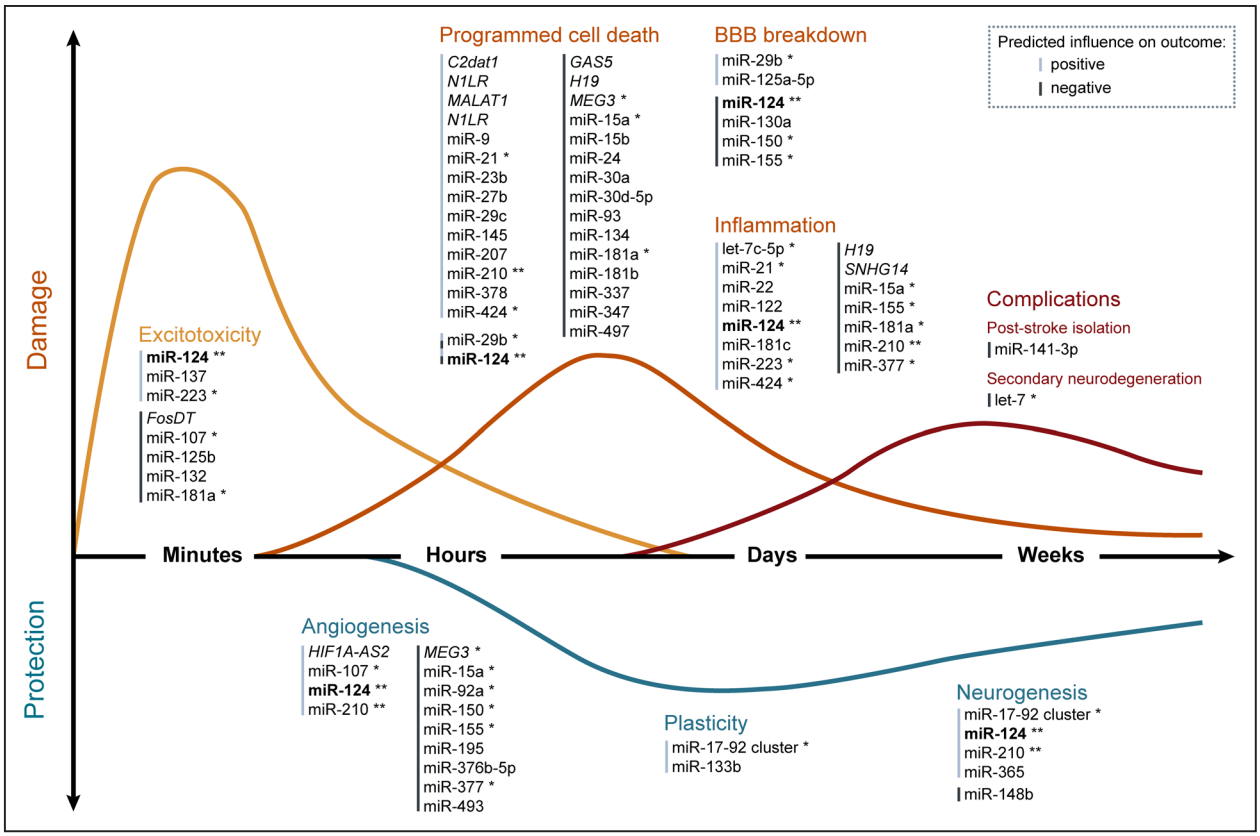


Figure 3. Complex and overlapping regulation of stroke pathophysiology by non-coding RNAs (ncRNAs). Listing of ncRNAs that have been linked to specific mechanisms of stroke pathophysiology. References are listed in the Figure in the online-only Data Supplement. Owing to its regulation of 6 pathophysiological processes, miR-124 is emphasized by bold type. *Involved in more than a single process; **involved in at least 4 processes. BBB indicates blood-brain barrier.

direct evidence for a relevant role of poststroke neuronal cell death mediated by endogenously released let-7 is still lacking.

Circulating ncRNAs as Biomarkers for the Diagnosis and Prognosis of Stroke

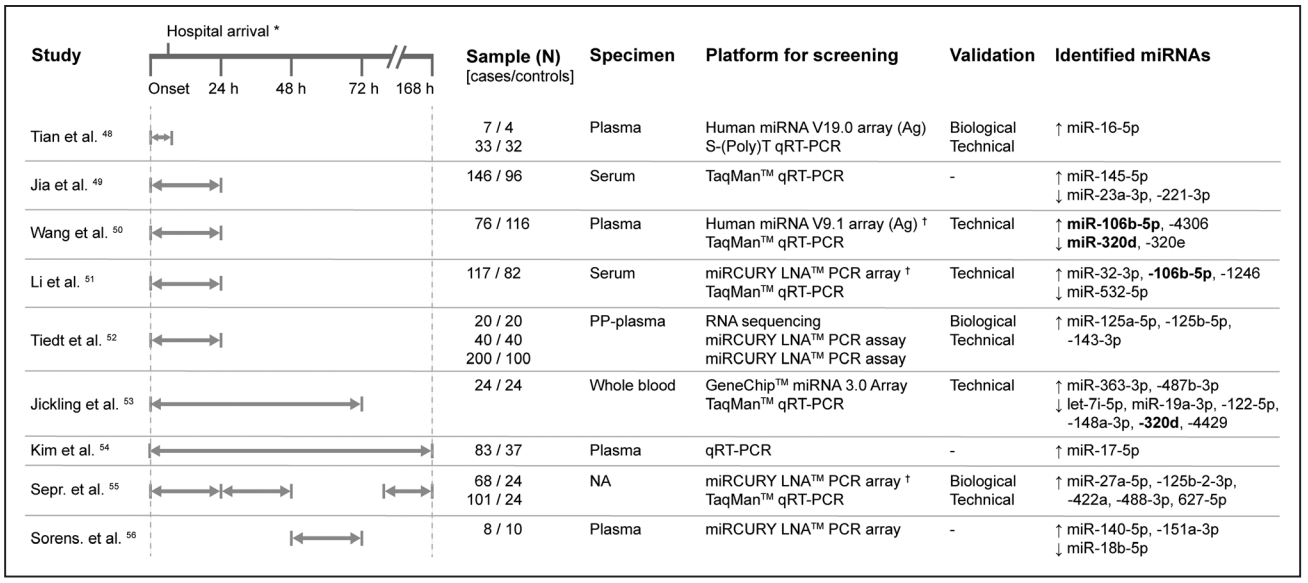
Owing to their cell type- and tissue-specific expression patterns and remarkable stability in peripheral blood ncRNAs have become a prime target for stroke biomarker research. The majority of studies focused on circulating miRNAs as potential biomarkers of acute ischemic stroke (IS; Figure 4). However, circulating ncRNAs have also been studied to detect patients at risk for incident or recurrent stroke and to predict stroke outcome.

In the largest study to date, Mick et al[10] profiled 2763 participants of the Framingham Heart Study for circulating small ncRNAs to determine whether extracellular RNAs are associated with incident or prevalent stroke. Using quantitative real-time polymerase chain reaction as a screening technique, they found miR-877-5p, miR-124-3p, miR-320d, and SNO1403 to be associated with prevalent stroke, whereas miR-656-3p and miR-941 were associated with incident stroke.[10] However, these findings still await confirmation in other populations.

Studies focusing on the acute phase of stroke have reported on a multitude of differentially regulated circulating ncRNAs (Figure 4). However, these findings were obtained using variable platforms for ncRNA detection and variable strategies for blood sampling in terms of both type of specimens (eg, whole blood versus platelet-poor plasma) and timing after stroke onset, all of which may influence the results. Only 2 studies explicitly restricted blood sampling to arrival in the emergency department (Figure 4),[48,52] thus minimizing the potential influence of early medication, such as heparin[57] or antiplatelet agents on the measurement of circulating miRNAs:

Tian et al[48] examined plasma samples of 40 patients presenting within 6 hours of symptom onset and from 27 healthy controls and applied a screening-and-validation approach that involved a microarray system in the screening phase. They identified 12 differentially expressed miRNAs, only one of which (miR-16-5p) could be validated in an independent sample with a sensitivity of 70% and a specificity of 87%.[48] Extending these results, another study found the combination of miR-16-5p and miR-124-3p to differentiate ischemic from hemorrhagic stroke, although this study included <100 patients, used a candidate rather than screening approach, lacked a validation sample, and did not provide data on sensitivity and specificity.[58] Of note, other studies have used miR-16-5p as an endogenous standard to normalize miRNA expression.[54]

We previously analyzed circulating miRNAs from platelet-poor plasma samples,[52] thereby limiting a potential influence of residual platelets in blood specimens on the profile of circulating miRNAs.[5] Other than previous studies on the acute phase after stroke, this study applied RNA sequencing for the screening stage. Through the use of 3 independent patient samples (overall N=492), we identified, validated (with quantitative real-time polymerase chain reaction), and



Study	Sample (N) [cases/controls]	Specimen	Platform for screening	Validation	Identified miRNAs
Tian et al. [48]	7 / 4 33 / 32	Plasma	Human miRNA V19.0 array (Ag) S-(Poly)T qRT-PCR	Biological Technical	↑ miR-16-5p
Jia et al. [49]	146 / 96	Serum	TaqMan™ qRT-PCR	-	↑ miR-145-5p ↓ miR-23a-3p, -221-3p
Wang et al. [50]	76 / 116	Plasma	Human miRNA V9.1 array (Ag) † TaqMan™ qRT-PCR	Technical	↑ **miR-106b-5p**, -4306 ↓ **miR-320d**, -320e
Li et al. [51]	117 / 82	Serum	miRCURY LNA™ PCR array † TaqMan™ qRT-PCR	Technical	↑ miR-32-3p, **-106b-5p**, -1246 ↓ miR-532-5p
Tiedt et al. [52]	20 / 20 40 / 40 200 / 100	PP-plasma	RNA sequencing miRCURY LNA™ PCR assay miRCURY LNA™ PCR assay	Biological Technical	↑ miR-125a-5p, -125b-5p, -143-3p
Jickling et al. [53]	24 / 24	Whole blood	GeneChip™ miRNA 3.0 Array TaqMan™ qRT-PCR	Technical	↑ miR-363-3p, -487b-3p ↓ let-7i-5p, miR-19a-3p, -122-5p, -148a-3p, **-320d**, -4429
Kim et al. [54]	83 / 37	Plasma	qRT-PCR	-	↑ miR-17-5p
Sepr. et al. [55]	68 / 24 101 / 24	NA	miRCURY LNA™ PCR array † TaqMan™ qRT-PCR	Biological Technical	↑ miR-27a-5p, -125b-2-3p, -422a, -488-3p, 627-5p
Sorens. et al. [56]	8 / 10	Plasma	miRCURY LNA™ PCR array	-	↑ miR-140-5p, -151a-3p ↓ miR-18b-5p

Figure 4. Challenges in interpreting studies investigating microRNAs (miRNAs) as biomarkers for acute ischemic stroke. Studies were selected that investigated the expression of at least 5 circulating miRNAs in patients within 72 h of symptom onset. miRNA nomenclature was unified to miRBase version 22. Highlighted miRNAs have been associated with ischemic stroke (IS) in more than a single study. Ag indicates Agilent; NA, no information available; PP-plasma, platelet-poor plasma; and qRT-PCR, quantitative real-time polymerase chain reaction. *Sampling on hospital arrival by Tian et al[48] and Tiedt et al[52]; † samples were pooled before the screening phase.

replicated a set of 3 miRNAs, miR-125a-5p, miR-125b-5p, and miR-143-3p (Figure 4). The sensitivity of this set to detect IS was superior to multimodal cranial computed tomography obtained for routine diagnostics.[52] Hence, while circulating ncRNAs might aid in the diagnosis of patients with IS, careful selection of the sampling strategy (timing and type of sample) and detection platforms remains critical.

Given their involvement in stroke pathogenesis (Figure 2) and repair mechanisms (Figure 3) expression levels of specific circulating miRNAs might further aid in the prediction of stroke recurrence and stroke outcome. Aside from studies investigating miRNA-based prediction of outcome as one among other aspects, a study in 329 patients with IS specifically focused on outcome. In a candidate-approach using plasma sampled within 72 hours of symptom onset miR-150-5p levels were found to improve risk classification for mortality at 90 days poststroke beyond traditional risk factors.[59] In contrast, the relation of circulating miRNAs and stroke recurrence has not been systematically addressed. However, the strong link between specific miRNAs and platelet function[5] emphasizes their potential to assist in identifying patients at risk for stroke recurrence.

Other than serum and plasma, analysis of whole blood might enable the identification of ncRNAs regulating the functional response of blood cells, such as the activation of leukocytes. Dykstra-Aiello et al[60] assessed the profile of lncRNAs in whole blood of 133 patients with IS and 133 healthy controls and found 396 differentially expressed lncRNAs, some of them mapping close to putative stroke risk genes. Hence, some of these lncRNAs might have relevance in mechanistically linking stroke risk loci to blood cell responses after stroke.

Opportunities and Challenges

As outlined above potential clinical applications of ncRNAs include (1) the utilization of expression levels of circulating ncRNAs for prognostication and diagnosis, that is to predict stroke, to diagnose stroke in the acute phase, and to predict outcome poststroke and (2) targeting their regulatory function for therapeutic purposes to prevent stroke and improve stroke outcome. However, translating these findings into clinical applications requires more work including validation of diagnostic and preclinical therapeutic findings in multicenter studies.

A major difficulty in interpreting previous biomarker studies originates from inconsistencies between the sets of circulating ncRNAs reportedly associated with stroke (Figure 4). Such inconsistencies are likely routed in the following factors, among others: (1) small sample sizes resulting in insufficient statistical power to capture true differences of hundreds of measured ncRNAs; (2) different recruitment strategies, inclusion and exclusion criteria resulting in differences in case mix, including age, sex, medication, and comorbidities, which are known to influence circulating ncRNA level variability[5]; (3) variable timing of blood sampling between and within studies, for example, when performed within the first 72 hours, a highly dynamic period covering largely variable stroke-related pathophysiological processes (Figure 3). Also, patients receive medication after hospitalization, among them low-molecular-weight heparin, which has been shown to interfere with the DNA polymerase during quantitative polymerase chain reaction[57]; (4) different blood specimens (serum versus plasma versus platelet-poor plasma). For instance, platelet aggregation during serum preparation may result in release of platelet miRNAs, trapping of miRNA within aggregates, and degradation of miRNA carrier proteins by proteases activated during clotting[5]; (5) different protocols for RNA isolation[61]; and (6) different detection platforms, such as quantitative real-time polymerase chain reaction, microarrays, and RNA sequencing, differing in sensitivity, accuracy, throughput, and their ability to detect novel miRNAs.[62]

Table 2. Advances Boosting ncRNA-Targeted Therapeutics Towards Clinical Application

Goal	Application
Identification of key ncRNAs	Multilevel omics, for example, RNA sequencing and untargeted proteomics
Improvement of cellular uptake and stability	Improvement of delivery vehicles
Selection of ncRNA candidates for human trials	Preclinical randomized controlled multicenter trials[69]

ncRNA indicates non-coding RNA.

To resolve the inconsistencies discussed above and facilitate the application of circulating ncRNA measurements to clinical diagnostic routine, a number of criteria should be met: (1) studies should be designed to target unmet clinical needs, which refers to any missing or inadequately performing component of a clinical pathway[63]; (2) potential methodological confounders should be carefully considered (see above and reviewed in Sunderland et al[5]); (3) reports should adhere to the guidelines for the reporting of diagnostic studies[64]; and (4) ideally, promising candidates should be validated in multicenter studies. Further, technological advances will overcome biases introduced by manual extraction methods and shorten processing times. Emerging techniques to quantify miRNA levels directly without the need to extract RNA and synthesize cDNA include the single molecule array platform SIMOA, employing the detection of single miRNAs by magnetic beads coupled to miRNA detection probes[65] and TORNADO (Theranostic One-Step RNA Detector), a technology based on electrocatalytic platinum nanoparticles.[66] Up to now, however, none of them has been applied in a clinical setting.

Other than diagnostic applications of ncRNAs, nucleic acid-based therapies have already been approved by the US Food and Drug Administration, an example being Mipomersen, an antisense oligonucleotide targeting apoB to treat patients with homozygous familial hypercholesterolemia.[67] Expectations are that the development of ncRNA-targeted therapeutics towards clinical applications in stroke will be facilitated by the following factors: (1) the advent of multilevel omics (eg, the application of parallel RNA sequencing and proteomics) will lead to the discovery of additional ncRNAs representing central nodes in dysregulated networks of stroke pathophysiology; (2) the improvement of delivery vehicles for miRNA-targeted therapeutics will enable targeted cellular uptake, high stability, and efficient crossing of the blood-brain barrier. Careful monitoring of potential systemic toxicity is necessary, illustrated by the finding that specific chemical modifications of antisense oligonucleotides to enhance uptake and stability in vivo have been shown to provoke platelet activation, aggregation, and thrombus formation[68]; and finally, (3) preclinical randomized controlled multicenter trials may be an informative step to select promising candidate ncRNAs before moving into patients (Table 2).[69] Additional challenges in lncRNA research include the presence of multiple transcript variants for individual lncRNAs, poor conservation, and the lack of a comprehensive and continuously updated database.

In conclusion, ncRNAs serve as central regulators of key pathological processes both before and after stroke. Circulating ncRNAs are dysregulated after stroke and might be used for diagnosis and prognosis to guide clinical decision-making. However, more work is needed to better delineate the involvement of specific ncRNAs in stroke and to exploit their utility for clinical applications.

Acknowledgments

We acknowledge Johannes Richers for the graphical illustration of Figures 1 and 2.

Sources of Funding

S. Tiedt was supported by a grant from the Deutsche Forschungsgemeinschaft (Munich Cluster for Systems Neurology [SyNergy]). M. Dichgans was supported by grants from the Deutsche Forschungsgemeinschaft (CRC 1123 [B3] and Munich Cluster for Systems Neurology [SyNergy]), the German Federal Ministry of Education and Research (BMBF, e:Med programme e:AtheroSysMed), the FP7/2007–2103 European Union project CVgenes@target (grant agreement No. Health-F2-2013–601456), the European Union Horizon2020 projects SVDs@target (grant agreement No. 66688) and Common Mechanisms and Pathways in Stroke and Alzheimer's Disease (CoSTREAM, grant agreement No. 667375), the Fondation Leducq (Transatlantic Network of Excellence on the Pathogenesis of Small Vessel Disease of the Brain), the Vascular Dementia Research Foundation, and the Jackstaedt Foundation.

Disclosures

None.

References

1. Encode Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489:57–74. doi: 10.1038/nature11247
2. Taft RJ, Pheasant M, Mattick JS. The relationship between non-protein-coding DNA and eukaryotic complexity. *Bioessays*. 2007;29:288–299. doi: 10.1002/bies.20544
3. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol*. 2014;15:509–524. doi: 10.1038/nrm3838
4. Flynt AS, Lai EC. Biological principles of microRNA-mediated regulation: shared themes amid diversity. *Nat Rev Genet*. 2008;9:831–842. doi: 10.1038/nrg2455
5. Sunderland N, Skroblin P, Barwari T, Huntley RP, Lu R, Joshi A, et al. MicroRNA biomarkers and platelet reactivity: the clot thickens. *Circ Res*. 2017;120:418–435. doi: 10.1161/CIRCRESAHA.116.309303
6. Wilhelm M, Schlegl J, Hahne H, Gholami AM, Lieberenz M, Savitski MM, et al. Mass-spectrometry-based draft of the human proteome. *Nature*. 2014;509:582–587. doi: 10.1038/nature13319
7. Thum T, Condorelli G. Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ Res*. 2015;116:751–762. doi: 10.1161/CIRCRESAHA.116.303549
8. Malik R, Chauhan G, Traylor M, Sargurupremraj M, Okada Y, Mishra A, et al; AFGen Consortium; Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium; International Genomics of Blood Pressure (iGEN-BP) Consortium; INVENT Consortium; STARNET; BioBank Japan Cooperative Hospital Group; COMPASS Consortium; EPIC-CVD Consortium; EPIC-InterAct Consortium; International Stroke Genetics Consortium (ISGC); METASTROKE Consortium; Neurology Working Group of the CHARGE Consortium; NINDS Stroke Genetics Network (SiGN); UK Young Lacunar DNA Study; MEGASTROKE Consortium; MEGASTROKE Consortium.

Multiancestry genome-wide association study of 520,000 subjects identifies 32 loci associated with stroke and stroke subtypes. *Nat Genet*. 2018;50:524–537. doi: 10.1038/s41588-018-0058-3
9. Holdt LM, Stahringer A, Sass K, Pichler G, Kulak NA, Wilfert W, et al. Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. *Nat Commun*. 2016;7:12429. doi: 10.1038/ncomms12429
10. Mick E, Shah R, Tanriverdi K, Murthy V, Gerstein M, Rozowsky J, et al. Stroke and circulating extracellular RNAs. *Stroke*. 2017;48:828–834. doi: 10.1161/STROKEAHA.116.015140
11. Verdura E, Hervé D, Bergametti F, Jacquet C, Morvan T, Prieto-Morin C, et al. Disruption of a miR-29 binding site leading to COL4A1 upregulation causes pontine autosomal dominant microangiopathy with leukoencephalopathy. *Ann Neurol*. 2016;80:741–753. doi: 10.1002/ana.24782
12. Feinberg MW, Moore KJ. MicroRNA regulation of atherosclerosis. *Circ Res*. 2016;118:703–720. doi: 10.1161/CIRCRESAHA.115.306300
13. Luo X, Yang B, Nattel S. MicroRNAs and atrial fibrillation: mechanisms and translational potential. *Nat Rev Cardiol*. 2015;12:80–90. doi: 10.1038/nrcardio.2014.178
14. Holdt LM, Hoffmann S, Sass K, Langenberger D, Scholz M, Krohn K, et al. Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. *PLoS Genet*. 2013;9:e1003588. doi: 10.1371/journal.pgen.1003588
15. Wu G, Cai J, Han Y, Chen J, Huang ZP, Chen C, et al. LincRNA-p21 regulates neointima formation, vascular smooth muscle cell proliferation, apoptosis, and atherosclerosis by enhancing p53 activity. *Circulation*. 2014;130:1452–1465. doi: 10.1161/CIRCULATIONAHA.114.011675
16. Sallam T, Jones M, Thomas BJ, Wu X, Gilliland T, Qian K, et al. Transcriptional regulation of macrophage cholesterol efflux and atherogenesis by a long noncoding RNA. *Nat Med*. 2018;24:304–312. doi: 10.1038/nm.4479
17. McDonald RA, Halliday CA, Miller AM, Diver LA, Dakin RS, Montgomery J, et al. Reducing in-stent restenosis: therapeutic manipulation of miRNA in vascular remodeling and inflammation. *J Am Coll Cardiol*. 2015;65:2314–2327. doi: 10.1016/j.jacc.2015.03.549
18. Canfrán-Duque A, Rotllan N, Zhang X, Fernández-Fuertes M, Ramírez-Hidalgo C, Araldi E, et al. Macrophage deficiency of miR-21 promotes apoptosis, plaque necrosis, and vascular inflammation during atherogenesis. *EMBO Mol Med*. 2017;9:1244–1262. doi: 10.15252/emmm.201607492
19. Jin H, Li DY, Chernogubova E, Sun C, Busch A, Eken SM, et al. Local delivery of miR-21 stabilizes fibrous caps in vulnerable atherosclerotic lesions. *Mol Ther*. 2018;26:1040–1055. doi: 10.1016/j.ymthe.2018.01.011
20. Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. *Science*. 2010;328:1566–1569. doi: 10.1126/science.1189123
21. Rayner KJ, Esau CC, Hussain FN, McDaniel AL, Marshall SM, van Gils JM, et al. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. *Nature*. 2011;478:404–407. doi: 10.1038/nature10486
22. Wu W, Xiao H, Laguna-Fernandez A, Villarreal G Jr, Wang KC, Geary GG, et al. Flow-dependent regulation of kruppel-like factor 2 is mediated by microRNA-92a. *Circulation*. 2011;124:633–641. doi: 10.1161/CIRCULATIONAHA.110.005108
23. Elmén J, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452:896–899. doi: 10.1038/nature06783
24. Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, et al. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. *Nat Med*. 2014;20:368–376. doi: 10.1038/nm.3487
25. Wagschal A, Najafi-Shoushtari SH, Wang L, Goedeke L, Sinha S, deLemos AS, et al. Genome-wide identification of microRNAs regulating cholesterol and triglyceride homeostasis. *Nat Med*. 2015;21:1290–1297. doi: 10.1038/nm.3980
26. Ramanujam D, Engelhardt S. Intercellular miRNA traffic. *Circ Res*. 2015;116:1726–1728. doi: 10.1161/CIRCRESAHA.115.306519
27. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature*. 2009;460:705–710. doi: 10.1038/nature08195
28. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, et al. MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. *EMBO Mol Med*. 2013;5:1017–1034. doi: 10.1002/emmm.201202318
29. Goedeke L, Rotllan N, Canfrán-Duque A, Aranda JF, Ramírez CM, Araldi E, et al. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. *Nat Med*. 2015;21:1280–1289. doi: 10.1038/nm.3949
30. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, et al; MICU Registry. MicroRNA-181b regulates NF-κB-mediated vascular inflammation. *J Clin Invest*. 2012;122:1973–1990. doi: 10.1172/JCI61495
31. Eken SM, Jin H, Chernogubova E, Li Y, Simon N, Sun C, et al. MicroRNA-210 enhances fibrous cap stability in advanced atherosclerotic lesions. *Circ Res*. 2017;120:633–644. doi: 10.1161/CIRCRESAHA.116.309318
32. Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, et al. MicroRNA-223 coordinates cholesterol homeostasis. *Proc Natl Acad Sci USA*. 2014;111:14518–14523.
33. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317–325. doi: 10.1038/nature10146
34. Ouimet M, Ediriweera HN, Gundra UM, Sheedy FJ, Ramkhelawon B, Hutchison SB, et al. MicroRNA-33-dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. *J Clin Invest*. 2015;125:4334–4348. doi: 10.1172/JCI81676
35. Lovren F, Pan Y, Quan A, Singh KK, Shukla PC, Gupta N, et al. MicroRNA-145 targeted therapy reduces atherosclerosis. *Circulation*. 2012;126(11 suppl 1):S81–S90. doi: 10.1161/CIRCULATIONAHA.111.084186
36. Wang D, Deuse T, Stubbendorff M, Chernogubova E, Erben RG, Eken SM, et al. Local microRNA modulation using a novel anti-miR-21-eluting stent effectively prevents experimental in-stent restenosis. *Arterioscler Thromb Vasc Biol*. 2015;35:1945–1953. doi: 10.1161/ATVBAHA.115.305597
37. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke*. 2008;39:959–966. doi: 10.1161/STROKEAHA.107.500736
38. Dharap A, Nakka VP, Vemuganti R. Effect of focal ischemia on long noncoding RNAs. *Stroke*. 2012;43:2800–2802. doi: 10.1161/STROKEAHA.112.669465
39. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005;433:769–773. doi: 10.1038/nature03315
40. Liu X, Li F, Zhao S, Luo Y, Kang J, Zhao H, et al. MicroRNA-124-mediated regulation of inhibitory member of apoptosis-stimulating protein of p53 family in experimental stroke. *Stroke*. 2013;44:1973–1980. doi: 10.1161/STROKEAHA.111.000613
41. Doeppner TR, Doehring M, Bretschneider E, Zechariah A, Kaltwasser B, Müller B, et al. MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. *Acta Neuropathol*. 2013;126:251–265. doi: 10.1007/s00401-013-1142-5
42. Harraz MM, Eacker SM, Wang X, Dawson TM, Dawson VL. MicroRNA-223 is neuroprotective by targeting glutamate receptors. *Proc Natl Acad Sci USA*. 2012;109:18962–18967.
43. Verma P, Augustine GJ, Ammar MR, Tashiro A, Cohen SM. A neuroprotective role for microRNA miR-1000 mediated by limiting glutamate excitotoxicity. *Nat Neurosci*. 2015;18:379–385. doi: 10.1038/nn.3935
44. Yan H, Rao J, Yuan J, Gao L, Huang W, Zhao L, et al. Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate ischemic neuronal death by targeting miR-21/PDCD4 signaling pathway. *Cell Death Dis*. 2017;8:3211. doi: 10.1038/s41419-017-0047-y
45. Caballero-Garrido E, Pena-Philippides JC, Lordkipanidze T, Bragin D, Yang Y, Erhardt EB, et al. In vivo inhibition of miR-155 promotes recovery after experimental mouse stroke. *J Neurosci*. 2015;35:12446–12464. doi: 10.1523/JNEUROSCI.1641-15.2015
46. Xi T, Jin F, Zhu Y, Wang J, Tang L, Wang Y, et al. MicroRNA-126-3p attenuates blood-brain barrier disruption, cerebral edema and neuronal injury following intracerebral hemorrhage by regulating PIK3R2 and Akt. *Biochem Biophys Res Commun*. 2017;494:144–151. doi: 10.1016/j.bbrc.2017.10.064
47. Lehmann SM, Krüger C, Park B, Derkow K, Rosenberger K, Baumgart J, et al. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat Neurosci*. 2012;15:827–835. doi: 10.1038/nn.3113

48. Tian C, Li Z, Yang Z, Huang Q, Liu J, Hong B. Plasma microRNA-16 is a biomarker for diagnosis, stratification, and prognosis of hyperacute cerebral infarction. *PLoS One*. 2016;11:e0166688. doi: 10.1371/journal.pone.0166688
49. Jia L, Hao F, Wang W, Qu Y. Circulating miR-145 is associated with plasma high-sensitivity C-reactive protein in acute ischemic stroke patients. *Cell Biochem Funct*. 2015;33:314–319. doi: 10.1002/cbf.3116
50. Wang W, Sun G, Zhang L, Shi L, Zeng Y. Circulating microRNAs as novel potential biomarkers for early diagnosis of acute stroke in humans. *J Stroke Cerebrovasc Dis*. 2014;23:2607–2613. doi: 10.1016/j.jstrokecerebrovasdis.2014.06.002
51. Li P, Teng F, Gao F, Zhang M, Wu J, Zhang C. Identification of circulating microRNAs as potential biomarkers for detecting acute ischemic stroke. *Cell Mol Neurobiol*. 2015;35:433–447. doi: 10.1007/s10571-014-0139-5
52. Tiedt S, Prestel M, Malik R, Schieferdecker N, Duering M, Kautzky V, et al. RNA-Seq identifies circulating miR-125a-5p, miR-125b-5p, and miR-143-3p as potential biomarkers for acute ischemic stroke. *Circ Res*. 2017;121:970–980. doi: 10.1161/CIRCRESAHA.117.311572
53. Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B, Liu D. microRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. *PLoS One*. 2014;9:e99283. doi: 10.1371/journal.pone.0099283
54. Kim JM, Jung KH, Chu K, Lee ST, Ban J, Moon J, et al. Atherosclerosis-related circulating microRNAs as a predictor of stroke recurrence. *Transl Stroke Res*. 2015;6:191–197. doi: 10.1007/s12975-015-0390-1
55. Sepramaniam S, Tan JR, Tan KS, DeSilva DA, Tavintharan S, Woon FP, et al. Circulating microRNAs as biomarkers of acute stroke. *Int J Mol Sci*. 2014;15:1418–1432. doi: 10.3390/ijms15011418
56. Sørensen SS, Nygaard AB, Nielsen MY, Jensen K, Christensen T. miRNA expression profiles in cerebrospinal fluid and blood of patients with acute ischemic stroke. *Transl Stroke Res*. 2014;5:711–718. doi: 10.1007/s12975-014-0364-8
57. Boeckel JN, Thomé CE, Leistner D, Zeiher AM, Fichtlscherer S, Dimmeler S. Heparin selectively affects the quantification of microRNAs in human blood samples. *Clin Chem*. 2013;59:1125–1127. doi: 10.1373/clinchem.2012.199505
58. Leung LY, Chan CP, Leung YK, Jiang HL, Abrigo JM, Wang de F, et al. Comparison of miR-124-3p and miR-16 for early diagnosis of hemorrhagic and ischemic stroke. *Clin Chim Acta*. 2014;433:139–144. doi: 10.1016/j.cca.2014.03.007
59. Scherrer N, Fays F, Mueller B, Luft A, Fluri F, Christ-Crain M, et al. MicroRNA 150-5p improves risk classification for mortality within 90 days after acute ischemic stroke. *J Stroke*. 2017;19:323–332. doi: 10.5853/jos.2017.00423
60. Dykstra-Aiello C, Jickling GC, Ander BP, Shroff N, Zhan X, Liu D, et al. Altered expression of long noncoding RNAs in blood after ischemic stroke and proximity to putative stroke risk loci. *Stroke*. 2016;47:2896–2903. doi: 10.1161/STROKEAHA.116.013869
61. McAlexander MA, Phillips MJ, Witwer KW. Comparison of methods for miRNA extraction from plasma and quantitative recovery of RNA from cerebrospinal fluid. *Front Genet*. 2013;4:83. doi: 10.3389/fgene.2013.00083
62. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med*. 2014;18:371–390. doi: 10.1111/jcmm.12236
63. Monaghan PJ, Lord SJ, St John A, Sandberg S, Cobbaert CM, Lennartz L, et al; Test Evaluation Working Group of the European Federation of Clinical Chemistry and Laboratory Medicine. Biomarker development targeting unmet clinical needs. *Clin Chim Acta*. 2016;460:211–219. doi: 10.1016/j.cca.2016.06.037
64. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig L, et al; STARD Group. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ*. 2015;351:h5527. doi: 10.1136/bmj.h5527
65. Cohen L, Hartman MR, Amardey-Wellington A, Walt DR. Digital direct detection of microRNAs using single molecule arrays. *Nucleic Acids Res*. 2017;45:e137. doi: 10.1093/nar/gkx542
66. McArdle H, Jimenez-Mateos EM, Raoof R, Carthy E, Boyle D, ElNaggar H, et al. "TORNADO" - Theranostic One-Step RNA Detector; microfluidic disc for the direct detection of microRNA-134 in plasma and cerebrospinal fluid. *Sci Rep*. 2017;7:1750. doi: 10.1038/s41598-017-01947-2
67. Raal FJ, Santos RD, Blom DJ, Marais AD, Charng MJ, Cromwell WC, et al. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2010;375:998–1006. doi: 10.1016/S0140-6736(10)60284-X
68. Flierl U, Nero TL, Lim B, Arthur JF, Yao Y, Jung SM, et al. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *J Exp Med*. 2015;212:129–137. doi: 10.1084/jem.20140391
69. Llovera G, Hofmann K, Roth S, Salas-Perdomo A, Ferrer-Ferrer M, Perego C, et al. Results of a preclinical randomized controlled multicenter trial (pRCT): anti-CD49d treatment for acute brain ischemia. *Sci Transl Med*. 2015;7:299ra121.

KEY WORDS: atherosclerosis ■ circulating microRNA ■ long non-coding RNA ■ microRNAs ■ stroke