Neurotherapeutics (2019) 16:299–313
https://doi.org/10.1007/s13311-019-00710-x

REVIEW

Nucleic Acid Therapies for Ischemic Stroke

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Published online: 11 January 2019

Abstract
Stroke remains a leading cause of disability and death worldwide despite significant scientific and therapeutic advances. Therefore, there is a critical need to improve stroke prevention and treatment. In this review, we describe several examples that leverage nucleic acid therapeutics to improve stroke care through prevention, acute treatment, and recovery. Aptamer systems are under development to increase the safety and efficacy of antithrombotic and thrombolytic treatment, which represent the mainstay of medical stroke therapy. Antisense oligonucleotide therapy has shown some promise in treating stroke causes that are genetically determined and resistant to classic prevention approaches such as elevated lipoprotein (a) and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Targeting microRNAs may be attractive because they regulate factors involved in neuronal cell death and reperfusion-associated injury, as well as neurorestorative pathways. Lastly, microRNAs may aid reliable etiologic classification of stroke subtypes, which is important for effective secondary stroke prevention.

Keywords Stroke · Neuroprotection · Nucleic acid · Prevention · Therapy · Review

Epidemiology of Stroke

The latest estimates from the Global Burden of Diseases, Injuries and Risk Factors Study 2016 (the most comprehensive source of comparable summary population health measures) have demonstrated a shift from communicable diseases to noncommunicable diseases as the leading causes for reduced disability-adjusted life-years (DALY; i.e., the sum of years of life lost due to poor health or disability) over the last 2 decades [1]. Among these, cerebrovascular diseases are now the second leading cause for DALYs [1]. These changes are driven by an increase in the incidence and prevalence of stroke in low- and middle-income countries resulting in a significantly higher stroke burden in these countries as compared to high-income countries [1]. Concerningly, stroke burden has particularly worsened among younger patients, with a startling 25% increase in the incidence of stroke among adults aged 20 to 64 years representing 31% of all people with incident stroke [2]. In 2010, there were 16.9 million incident cases of stroke (~70% ischemic strokes and ~30% hemorrhagic strokes) worldwide [2] and approximately 800,000 people suffer a stroke in the USA each year [3, 4]. Despite significant scientific and therapeutic advances, stroke remains the fifth leading cause of death in the USA and there has been a recent flattening, and even increase, in death rates among all age groups [4, 5]. Stroke-associated socioeconomic costs are immense, and given our aging society, it has been estimated that the total direct medical stroke-related costs will more than double from $36.7 billion to $94.3 billion from 2015 to 2035 [4]. Hence, there is a critical need to improve stroke prevention and treatment. In the following pages, we will provide a narrative overview of select, promising strategies that leverage nucleic acid therapeutics to improve stroke care through the 3 main pillars prevention, acute treatment, and recovery (Table 1 summarizes key aspects of the discussed agents) [6, 7]. In this

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13311-019-00710-x) contains supplementary material, which is available to authorized users.

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Table 1 Summary of discussed nucleic acid therapies

Molecule	Mode of action	Benefits/adverse effects	Developmental stage
Antithrombotic therapy for primary and secondary stroke prevention			
ARC1779 (aptamer)	Blocks platelet activation through vWF-GPIb interaction	Dose- and concentration-dependent inhibition of vWF activity and platelet function / No major bleeding events or side effects reported	Phase I
		Decreased rate of CEA-related embolic events and radiographic defined stroke / Increased bleeding events	Phase II
Ch-9.14-T10 (aptamer)	vWF-targeting aptamer (similar to ARC1779)	Attenuated ferric chloride induced carotid artery thrombosis in the mouse	Preclinical (*in vivo*)
REG1 (aptamer–antidote system)	Blocked conversion of factor X to Xa	Controllable anticoagulation in healthy volunteers / No major bleeding events or side effects reported	Phase I
		Trend towards fewer ischemic complications among patients undergoing cardiac catheterization / Serious allergic reactions observed	Phase IIb
		Decreased in-stent thrombosis after PCI. / Increased bleeding risk and severe allergic reactions to the PEG moiety	Phase III
Atherogenic stroke prevention			
Mipomersen (ASO)	Decreased hepatic protein synthesis of apoB-100	Decreased major cardiac events / Hepatotoxicity	Phase III (available under a REMS)
IONIS-APO$_{(a)Rx}$ (ASO)	Decreased hepatic protein synthesis of apoB-100	Dose-dependent Lp$_{(a)}$-reduction / No serious adverse events reported	Phase I
Prevention in inherited stroke syndromes			
NOTCH3-targeting ASO	Altered pre-mRNA splicing to correct the number of cystein molecules	NOTCH-3 protein was functional and did not form pathologic aggregates	Preclinical (*in vitro*)
Acute ischemic stroke therapy: thrombolysis			
tPA binding RNA aptamer	Inhibited tPA-LRP-1 complex formation	Attenuated BBB disruption and neuronal excitotoxicity without affecting tPA's fibrinolytic activity	Preclinical (*in vitro*)
Acute ischemic stroke therapy: neuroprotection			
miR-215	Overexpression of miR215 thought to attenuate upregulation of Act-1 and Il-17	Inhibited apoptosis and autophagy as well as attenuated infarct volume and functional deficits	Preclinical (*in vitro*, *in vivo*)
Acute ischemic stroke therapy: inflammation			
miR-1906	Suppressed TLR-4	Decreased the inflammatory response	Preclinical (*in vivo*)
miR-21 mimic	Inhibited MAPK-signaling through MAP2K3	Attenuated BBB disruption, infarct extent, and functional deficit severity	Preclinical (*in vivo*)
anti-miR-613	Putative molecular target not reported	Decreased lipid peroxidation and ROS formation	Preclinical (*in vitro*)
Spatholobus suberectus	Decreased miR494, lead to upregulating of *sox8* mTOR/MAPK	Improved functional outcomes after stroke	Preclinical (*in vitro*)
AQP-4 targeting siRNA	AQP-4 knockdown lead to *c-fos* and NGFI-B upregulation	Proof of principle that inhibiting AQP-4 affects astrocyte viability	Preclinical (*in vitro*)
Acute ischemic stroke therapy: recovery			
miR-17-92	Inhibited phosphatase and tensin homolog target genes with resultant phosphorylation of downstream proteins	Increased neuronal differentiation, plasticity, and enhanced neurological recovery	Preclinical (*in vivo*)

Table 1 (continued)

Molecule	Mode of action	Benefits/adverse effects	Developmental stage
anti-miR-155	Increased expression of SMAD 5, Rictor, eNOS, and Rheb	Increased microvascular and BBB integrity, attenuated neuronal injury, and increased functional recovery	Preclinical (*in vivo*)
Ischemic stroke biomarkers circRNA-284-to-miR-221 ratio		Increased ratio identified patients with symptomatic carotid disease	Observational

Act-1 = nuclear factor (NF)-κB activator; apoB-100 = apolipoprotein B; AQP-4 = aquaporin-4; ASO = antisense oligonucleotide; BBB = blood brain barrier; CEA = carotid endarterectomy; eNOS = endothelial nitric oxide synthase; IONIS-$APO_{(a)Rx}$ = antisense apolipoprotein A inhibitor, Il-17 = interleukin 17, LRP-1 = low-density lipoprotein receptor-related protein-1, Lp(a) = lipoprotein(a), mTOR = mechanistic target of rapamycin, MAPK = mitogen-activated protein kinases, MAP2K3 = mitogen-activated protein kinase kinase 3, *NGFI-B* = nerve growth factor inducible protein-B, NOTCH3 = neurogenic locus notch homolog protein 3, REG1 = pegnivacogin + anivamersen, REMS = risk evaluation and mitigation strategy, Rheb = ras homolog enriched in brain, Rictor = rapamycin-insensitive companion of mammalian target of rapamycin, ROS = reactive oxygen species, SMAD 5 = mothers against decapentaplegic homolog 5, sox8 = sex determining region Y-box 8, TLR-4 = toll like receptor-4, tPA = tissue-type plasminogen activator, vWF-GPIb = von Willebrand factor–glycoprotein Ib interaction

chapter, we will focus on ischemic stroke because the majority of nucleic acid therapies have been tested in this stroke type and because ischemic stroke represents the most common stroke form. For details regarding nomenclature, chemistry, and challenges in delivering nucleic acid therapeutics to the central nervous system, we refer the interested reader to the companion chapters in this special edition.

Stroke Prevention

Antithrombotic Therapy for Primary and Secondary Stroke Prevention

The 3 major ischemic stroke mechanisms include cardioembolism, large artery atherosclerosis, and cerebral small vessel disease related pathology [8, 9]. The majority of all ischemic strokes are caused by arterial thromboembolism. Hence, prophylaxis with antithrombotics (i.e., anticoagulants and antiplatelet agents) is the mainstay of medical therapy to reduce stroke risk. However, despite the proven benefit of antithrombotic therapy, many recurrences are not prevented. Additionally, the use of antiplatelets and anticoagulants carries the risk of bleeding complications particularly if used in combination [10–13]. Accordingly, there is a need to refine antithrombotic regimens and to develop novel agents to increase benefit while attenuating hemorrhage risk.

One promising approach for inhibiting platelet function is to block the interaction between von Willebrand factor (vWF) and the platelet receptor glycoprotein 1b (GP1b) to minimize recruitment, activation, and aggregation of platelets at an injured arterial wall. Preclinical studies found that inhibition of GPIb or absence of vWF confers profound antithrombotic effects as well as attenuates infarct size in a mouse transient middle cerebral artery occlusion stroke model without increasing the risk for hemorrhagic transformation of the brain infarct [14–17]. Under pathological conditions (that cause high shear force in the arterial circulation), vWF is activated through physical deformation that exposes its A1 domain and enables binding to the GP1b receptor resulting in thrombosis [18].

The first aptamer against vWF was ARC1172, a DNA oligonucleotide that bound to the A1-domain of vWF [19]. Subsequently, the anti vWF RNA/DNA hybrid aptamer ARC1779 was developed. ARC1779 binds to the A1 domain of activated vWF, blocking the interaction of vWF with GP1b on platelets, inhibiting the vWF-mediated pathological thrombosis, and leaving the coagulation system and other pathways of platelet activation intact [20]. A clinical phase 1 study in healthy volunteers demonstrated that ARC1779 dose-dependently reduced vWF activity and platelet function. A subsequent small randomized, double-blind, placebo-controlled phase 2 trial in patients undergoing carotid endarterectomy (CEA) demonstrated that patients treated with

ARC1779 had significantly later occurrence of postoperative embolic signals as detected by transcranial Doppler during the 3 h of monitoring. Moreover, the number of patients without any embolic signals was significantly lower in the treatment group and there was a trend towards overall fewer embolic signals in ARC1779 patients. Also, none of the 8 subjects in the ARC1779 group who underwent brain MRI had evidence of postoperative ischemic stroke as compared to 2 of 5 patients in the placebo group. Lastly, the number of clinical overt strokes was similar between groups (one each) [21]. Bleeding events were more common in ARC1779-treated subjects [21] for which reason phase II and III trials will be required to establish safety and efficacy of ARC1779 for ischemic stroke prevention particularly in the perioperative setting. In this respect, a possible approach to mitigate hemorrhage risk in the surgical setting as well as to control bleeding complications in patients treated with aptamers targeting vWF is to use an aptamer inhibitor. It has been shown that the RNA aptamer Ch-9.14-T10 maintained arterial patency in the mouse ferric chloride-induced carotid artery thrombosis model [22]. The authors demonstrated that surgically challenged mice (tail transsection) treated with Ch-9.14-T10 dose-dependently exhibited significantly enhanced bleeding as compared with control mice. However, by using a complementary antidote oligonucleotide based on the sequence of aptamer Ch-9.14-T10, they were able to reverse vWF aptamer activity both *in vitro* and *in vivo*, resulting in substantial attenuation of bleeding in surgically challenged mice (with similar blood loss as in control animals) [22].

Long-term oral anticoagulation remains the mainstay for preventing ischemic stroke in patients at high risk for cardioembolism such as in the setting of atrial fibrillation, the most common pathological arrhythmia [23–25]. Although stroke prevention with oral anticoagulation is key to AF treatment [26–29], up to 40% of treatment-eligible older atrial fibrillation patients are untreated due to complex decision-making [30–40]. It is particularly challenging for clinicians to advise frail patients about anticoagulation given their high risk for both ischemic stroke and anticoagulation-related bleeding [41–44]. Currently available oral anticoagulants include vitamin K antagonists (e.g., warfarin) and non-vitamin K oral anticoagulants (NOAC) including the direct thrombin inhibitor dabigatran and factor Xa inhibitors such as apixaban, rivaroxaban, and edoxaban [45]. Aptamer–antidote systems have been developed as a fundamentally attractive regimen to achieve rapid and selective anticoagulation with the ability for graded reversal in patients requiring safe and effective anticoagulation including in the setting of procedures. One of these systems is REG1, which consists of an active anticoagulant (pegnivacogin, RB006) and a complementary oligonucleotide antidote (anivamersen, RB007) that neutralizes the anticoagulant effect as needed, serving as a molecular "on–off" switch [46, 47]. Because anivamersen restores hemostatic capacity by preventing the association of pegnivacogin with factor IXa, the maximal generated levels of factor IXa are limited to pre-existing levels of native factor IX/IXa assuaging concerns that reversal results in exceeding intrinsic factor IXa activity and trigger thrombosis [46, 48]. Following a comprehensive preclinical development program, REG1 was tested as the first-in-human aptamer-based direct factor IXa inhibitor [47–50]. To achieve prolonged duration of effect, pegnivacogin was chemically stabilized by conjugation to a 40-kDa polyethylene glycol (PEG) carrier. Pegnivacogin selectively blocks the conversion of factor X to factor Xa. In the first phase 1a study that enrolled 85 healthy volunteer subjects, REG1 was overall well tolerated and adverse bleeding events were similar to placebo (mostly consisting of minor bleeding and ecchymoses at the intravenous access site) [47]. Notably, one pegnivacogin-treated patient developed transient speech impairment, mood alteration, confusion, and ptosis that spontaneously resolved. However, given the patient's personal history (drug abuse), circumstances of symptom occurrence (emotional exchange with study staff), and absent overt mechanistic link, it was uncertain whether the event was caused by the study drug [47]. In a second phase 1 trial, testing repeat dosing of REG1 components in 39 healthy volunteers, no significant adverse events were observed [51]. In anticipation of phase 2 trials of revascularization therapy, the subsequent phase 1b study enrolled 50 subjects with stable coronary artery disease on maintenance single or dual antiplatelet therapy to determine the clinical safety and pharmacodynamic profiles of REG1 [46]. Similar to the phase 1 studies in healthy volunteers, REG1 was overall well tolerated without major bleeding or other serious adverse events or signs of acute encephalopathy [46]. Transient cutaneous reactions (flushing and/or pruritus) were noted in 2 subjects within a few minutes after drug injection. Pegnivacogin resulted in dose-dependent, rapid-onset, and durable anticoagulation that was rapidly reversed with anivamersen. The RADAR phase IIb trial tested different levels of reversal of pegnivacogin by anivamersen compared with heparin in 640 patients with acute coronary syndrome undergoing cardiac catheterization. This trial found that with at least 50% reversal of pegnivacogin, bleeding rates were similar to heparin (enrollment in the 25% reversal arm was stopped due to excess bleeding) and there was a trend towards less frequent acute ischemic complications in REG1 as compared to heparin anticoagulation [52]. However, 3 patients developed serious unexplained allergic reactions and study enrollment was terminated after the third event. Based on the encouraging results of a reduction in the incidence of ischemic events to 3.0% compared with 5.7% in the heparin arm REGULATE-PCI, a large clinical phase III trial commenced with the goal to determine the efficacy of REG1 *versus* bivalirudin for percutaneous coronary intervention in more than 13,000 patients [53]. In light of the observed

allergic reactions in the prior trials, specific guidelines were provided to investigators and patients. The data and safety monitoring board reviewed in real time and periodically all serious allergic events. Although stent thrombosis by day 30 occurred less frequently with REG1, there was no difference in the primary efficacy endpoint (death, myocardial infarction, stroke, or unplanned target lesion revascularization by day 3). Secondary composite or individual efficacy endpoints and bleeding were more frequent among patients receiving REG1. However, this study was halted following enrollment of approximately 3200 participants after 10 serious allergic reactions to pegnivacogin occurred (including 1 fatality) [53]. This observation prompted additional *post hoc* studies to determine the cause of these adverse events. These found a strong correlation between the incidence of allergic reactions and the presence of pre-existing circulating anti-PEG antibodies. Moreover, patients from REGULATE-PCI who experienced the most severe reaction had the highest levels of pre-existing anti-PEG antibodies [54, 55]. It was concluded that the PEG moiety and not the aptamer component of pegnivacogin was responsible for the severe allergic reactions [54, 55]. Although further clinical development of pegnivacogin has been discontinued, the overall results gained from the clinical trials provided highly insightful information for future drug and trial design. It also showed that aptamer-based inhibition of factor IXa can provide effective anticoagulation providing the rational for ongoing efforts to develop novel aptamer-based anticoagulant strategies [56].

Atherogenic Stroke Prevention

Prospective longitudinal studies have established the importance of major atherogenic risk factors including hypertension, diabetes mellitus, obesity, obstructive sleep apnea, and smoking [57]. Dyslipidemia is another well-established modifiable risk factor that contributes to the development of cerebrovascular disease and stroke. The use of HMG-CoA reductase inhibitors (statins) is recommended by the American Heart Association to reduce the risk of stroke and cardiovascular events particularly in patients where the stroke was related to atherosclerotic disease [57, 58]. These recommendations are based on the SPARCL trial that demonstrated a 16% relative risk reduction of recurrent strokes in patients treated with 80 mg atorvastatin [59]. However, it is interesting to note that a meta-analysis of 45 prospective studies including ~ 450,000 subjects did not find a significant association between total serum cholesterol level and stroke incidence [60]. Similarly, observations from the Framingham Heart Study indicated that a low high-density lipoprotein (HDL) but not total serum cholesterol and low-density lipoprotein (LDL) cholesterol relate to stroke risk [61]. Nevertheless, total serum cholesterol and LDL cholesterol have been shown to directly contribute to extracranial carotid artery atherosclerosis whereas HDL cholesterol has been found to exert protective effects [62–64]. Accordingly, it remains presently unclear whether the beneficial effects of statins are mediated through its proven LDL-lowering properties, through anti-inflammatory, neuroprotective, and neurorestorative attributes, or by targeting other (clinically less frequently assessed) lipoprotein fractions.

For example, a meta-analysis suggested that atorvastatin may lower lipoprotein (a) (Lp(a)) [65]. Lp(a) is a unique LDL-like particle that is comprised of a moiety essentially identical to LDL, which is covalently linked to the distinguishing protein component apolipoprotein(a) [66–69]. Lp(a) is an attractive target for reducing stroke risk through nucleic acid therapies because (1) Lp(a) concentrations in the atherogenic range are highly prevalent, affecting an estimated 20 to 30% of the worldwide population; (2) Lp(a) has a proven causal association with cardiovascular diseases and ischemic stroke [70–77]; and (3) plasma Lp(a) concentrations are largely determined by genetic factors confined to the apo(a) encoding gene LPA [78], which renders it resistant to dietary and other lifestyle modifications as well as treatment with classic lipid-lowering agents (such as niacin and statins) [66, 79]. Furthermore, though several trials demonstrated feasibility to modestly lower Lp(a) concentrations using classic lipid-lowering agents, none have provided evidence that the achieved degree of Lp(a) reduction leads to reduced cardiovascular events and stroke [76, 79, 80]. Yet, proof of principle that Lp(a) lowering in maximally treated patients may improve outcome stems from a prospective observational multicenter study demonstrating that lipid apheresis effectively reduced the frequency of cardiovascular and cerebrovascular events over a follow-up period of 2 years [81].

Antisense oligonucleotide (ASO) therapy has shown some promise in treating elevated Lp(a). For example, plasma Lp(a) levels have been consistently and significantly reduced with mipomersen (ISIS 301012). Mipomersen is a second-generation antisense oligonucleotide that specifically binds to the apolipoprotein B-100 mRNA, which was the first agent to enter clinical trials utilizing an antisense mechanism for reducing the production of apolipoprotein B. By inhibition of messenger ribonucleic acid translation, mipomersen blocks the hepatic protein synthesis resulting in dose-dependent lowering of the concentration of the apoB-100 containing atherogenic lipoproteins including Lp(a) in patients with varying extents of hyperlipidemia who are at high risk for cerebrovascular events (such as in patients with familial hypercholesterolemia and on the background of treatment with statins and other conventional lipid-lowering drugs) [82–89]. Specifically, several phase III clinical trials have shown that mipomersen lowers Lp(a) by approximately 17 to 30% [85–89]. Importantly, an individual patient analysis of subjects that had participated in one of the 3 phase III trials of the mipomersen program [85–87] found a significant reduction in major adverse cardiac events from

25.7 of 1000 patient-months of follow-up before mipomersen treatment to 3.9 of 1000 patient-months of follow-up during approximately 24 months of mipomersen treatment (odds ratio 0.053 [95% CI, 0.016-0.168], $P < 0.0001$) [88]. Nevertheless, despite the compelling 84% relative reduction in major cardiovascular events, it needs to be emphasized that mipomersen treatment related to a significant reduction in all atherogenic lipid fractions. Thus, it remains uncertain to what extent the lowering of Lp(a) contributed to the risk reduction. Moreover, major predictors of mipomersen-associated Lp(a) reduction were white race and lower baseline values, which is of clinical importance because approximately 30% of whites, yet 60% to 70% of blacks, have elevated Lp(a) levels of > 30 mg/dL [90]. Lastly, despite the proven efficacy in lowering Lp(a) and other atherogenic lipids, mipomersen possesses a significant side effect profile including risk for hepatotoxicity, for which reason it is unlikely to find wide acceptance for the specific treatment of isolated elevations of Lp(a) (and for which reason it is restricted for use in homozygous familial hypercholesterolemia through a risk evaluation and mitigation strategy program in the USA and was rejected by European Medicines Agency) [79, 91]. Nevertheless, subsequent investigations into optimized ASOs for treatment of increased Lp(a) identified IONIS-APO(a)$_{Rx}$, which achieved a dose-dependent mean Lp(a) reduction of 78% in healthy volunteers [70]. In this first phase I study, IONIS-APO(a)$_{Rx}$ did not cause any serious or severe adverse events and there were no significant changes in liver function assays. Common less severe events included mild injection site reactions as well as flu-like symptoms [70]. The results from this study provide the rationale for future clinical trials to determine whether lowering Lp(a) plasma concentrations reduces cardiovascular events including ischemic stroke.

Prevention in Inherited Stroke Syndromes

The majority of ischemic strokes are multifactorial in nature, and any genetic contributions are likely the result from multiple risk alleles each with small effects [92]. Nevertheless, a small subset of ischemic strokes have monogenic causes, posing a substantial challenge for clinicians because standard approaches to risk factor modification and secondary prevention measures, while partially beneficial, are not sufficient in preventing disease progression [92]. A prominent example is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). It is caused by mutations in the *NOTCH3* gene on chromosome 19 first reported in European families. Today, CADASIL has been reported from all continents and in hundreds of families of European, American, African, and Asian descent. It is estimated to inflict 1 in 25,000 to 50,000 people. Patients with the disease tend to be middle aged who classically develop migraine with aura as the earliest clinical manifestation. While migraine with aura may be the prominent symptom in some families, stroke is the most frequent clinical manifestation over a patient's lifetime, with approximately two thirds of symptomatic subjects having had a stroke/transient ischemic attack. Additional symptoms include mood disturbances as well as dementia relating to chronic, progressive brain injury due to white matter disease and strokes. Cognitive disturbances are typically in multiple domains including visuospatial and speech as well as memory. Specific imaging findings include MRI hyperintensities in the bilateral temporal horns, the external capsule, and the corpus callosum indicating extensive cerebral small-vessel disease-related pathology. Whereas imaging findings and clinical presentation are suggestive, diagnosis is made through genetic testing or skin biopsy, revealing loss of the media tunica and fibrosis of the adventitia, with cytoplasmic inclusions in the vascular smooth muscles. It has been shown that mutations in the *NOTCH3* gene lead to cell surface aggregates, yet the intracellular cytoskeleton is affected. Ultimately, the vascular smooth muscles are unable to contract causing aberrancies in autoregulation of small vessels in the central nervous system [93]. To date, there are no specific treatments for this devastating disease and therapy rests on supportive measures [94–96]. CADASIL treatment has been met with difficulties as *NOTCH3* is ubiquitously expressed and functions in multiple organ systems, even at early stages of embryogenesis [97, 98]. Several ongoing trials testing the use of antibodies in order to alter the activation of notch-3 cascade have been promising [98, 99]. Silencing the gene through shRNA, transfected via a lentivirus, causes similar pathology [93]. In a proof-of-concept experiment, smooth muscle cells from CADASIL patients were treated using ASOs to alter the pre-mRNA splicing and correct the even number of cystein molecules at the extracellular epidermal growth factor-like repeat (EGFr) domain. The remaining notch-3 protein was functional and did not form pathologic aggregates [100]. Although *in vivo* validation of this approach is warranted, nucleic acid-based therapies may be an exciting novel approach to treating this as well as other monogenic stroke causes and related sequelae.

Acute Ischemic Stroke Therapy

The brain relies on a constant supply of oxygen and high-energy substrates (predominantly glucose) to satisfy its high metabolic demands to maintain functional and structural integrity. Simplistically, focal brain ischemia results from occlusion or stenosis of a brain supplying vessel through embolism of material originating elsewhere in the vasculature or from *in situ* vascular pathology such as atherothrombosis [101]. Vascular occlusion and thus interrupted delivery of substrates to a vascular territory of the brain quickly result in ischemia that progresses to irreversible infarction if blood flow is not

reinstated in a timely fashion [102]. It has been estimated that with each minute of ischemia, 1.9 million neurons are permanently lost [103]. Accordingly, rapid reperfusion of the ischemic but not yet infarcted tissue is paramount to mitigate brain injury. Indeed, the only proven efficacious acute stroke treatment strategies are based on this principle. Although there are a multitude of preclinical studies that have utilized nucleic acid therapeutics to investigate pathophysiology and establish proof of principle therapeutic targets, there are presently no well-developed clinical nucleic acid therapeutic programs similar to those presented in the preceding sections on stroke prevention. Therefore, we will focus on general principles of acute stroke therapy and opportunities for intervention supported by discussion of select targets of potential interest.

Thrombolysis

The most commonly used therapy that is effective and safe for acute ischemic stroke therapy is intravenous recombinant tissue plasminogen activator (tPA). Since its initial approval for treatment of patients within a narrow time window of 3 h from symptom onset [104–106], its indication has been safely expanded for use in the 4.5-h time window in patients selected on additional clinical criteria [107], and more recent studies demonstrate the possibility to treat even longer after symptom onset by using advanced neuroimaging criteria [108]. Nevertheless, despite proven benefit and overall safety, thrombolysis with tPA is only partially effective in many patients and it increases the risk for intracranial hemorrhage [109–111]. Furthermore, preclinical studies indicated that tPA may exert neurotoxic properties and exacerbate ischemic lesions via several distinct pathways [112–114]. Hence, minimizing tPA-mediated toxicity is a potential strategy to increase the benefit-to-risk ratio. A recently proposed strategy is to target the interaction of tPA with the low density lipoprotein receptor related protein-1 (LRP-1) [115], which is a transmembrane receptor expressed on several cell types including neurons, vascular endothelial cells, pericytes, smooth muscle cells, and astrocytes. The LRP-1 interaction with tPA appears to be an important mediator of adverse effects after tPA-mediated thrombolysis. For example, LRP-1-dependent blood–brain barrier (BBB) disruption as well as hemorrhagic transformation has been shown after tPA administration [114, 116]. Indeed, tPA-binding RNA aptamers have been developed that inhibit the tPA/LRP-1 complex formation and subsequent receptor-mediated endocytosis of tPA without substantially affecting the fibrinolytic properties of tPA *in vitro* [115]. These observations provide proof of principle that aptamer technology can be leveraged to attenuate potential tPA-mediated tissue toxicity while preserving its beneficial thrombolytic properties. This observation opens the door to developing novel strategies to increase the safety of thrombolytic agents.

Neuroprotection

After the onset of focal brain ischemia, the brain region with impaired cerebral blood flow contains subregions that progress to irreversible infarction at differing amounts of time depending primarily upon the severity of the initial cerebral blood flow decline, but metabolic factors and temperature can affect the rapidity of infarct development [117]. The ischemic region that is already infarcted at any given time point after the onset of ischemic stroke is the ischemic core, whereas the ischemic region at risk for becoming infarcted over time is known as the ischemic penumbra. The ischemic penumbra is the tissue target of acute stroke therapies, mediated either by reperfusion or neuroprotection, because therapeutic intervention can salvage ischemic tissue destined to become infarcted and thereby preserve functional capacity, leading to a better clinical outcome [118]. The cellular consequences of reduced or absent blood flow to the brain are manifold and referred to as the ischemic cascade [119].

However, in contrast to the increasingly positive outcomes with pharmacological (i.e., tPA-treatment) or mechanical thrombolysis in ischemic stroke [104–108, 120–128], clinical trials testing putative neuroprotective drugs targeting the various key factors in the ischemic cascade have been disappointing. Although well over 1000 compounds have shown promise in preclinical studies as neuroprotective agents, none of them were found to be effective in phase III clinical trials in which they were compared to placebo [129, 130]. Many reasons were identified for the failure of translation of monotherapy neuroprotection from successful animal models in clinical trials, which led to a number of suggestions as to how to improve neuroprotective drug testing [131–136]. In addition, the major advances in reperfusion therapy for acute ischemic stroke require reassessment of neuroprotection. This should now be viewed as an adjunctive therapy to be employed before, during, or after systemic thrombolysis and mechanical thrombectomy rather than standalone treatment because the convincing efficacy of reperfusion therapy would make it unethical to withhold these proven therapies. Several scenarios for using neuroprotection in conjunction with reperfusion can be envisioned including the expansion of the time window for definite reperfusion ("penumbral freezing"). Proof of principle for extending penumbral survival, and thus the therapeutic time window for tPA-mediated reperfusion, has been shown with several pharmacological interventions as well as with inhaled nitric oxide and high-flow oxygen therapy to function as neuroprotective gas treatments [137–139]. Such observations raise the intriguing possibility of testing neuroprotective drugs as a way to keep the ischemic core from expanding and the ischemic penumbra from

shrinking prior to definite reperfusion therapy, for example, as patients are being transported to tertiary stroke centers for treatment.

In this respect, nucleic acid therapies targeting microRNA (miRNA) may be attractive because distinct miRNA expression patterns have been found in patients with ischemic stroke with both up- and downregulation of miRNAs as assessed in the blood [140], and also because miRNAs have been implicated in the regulation of factors involved in neuronal cell death as well as reperfusion-associated injury [141–143]. For example, miR-215 has been found upregulated in preclinical models of neuronal ischemic injury and overexpression of miR-215 inhibited apoptosis and autophagy *in vitro*, as well as attenuated the infarct volume and improved functional deficits in a mouse ischemic stroke model [144]. It has been posited that decreased levels of miR-215 in ischemic conditions lead to upregulation of nuclear factor (NF)-κB activator (Act)1 based on bioinformatics modeling, which ultimately leads to activation of interleukin 17 (Il-17) causing enhanced apoptosis and autophagy.

Targeting Inflammation

Inflammation plays a pivotal role at all stages of cerebral ischemia and is initiated via activation of platelets, complement, and endothelial cells. Leukocytes are subsequently activated by the release of cytokines and adhesion molecules, which includes tumor necrosis factor alpha among others. The humoral response is subsequently propagated by all cell types in the neurovascular unit, including endothelial cells, glia (astrocytes, microglia, oligodendrocytes), and neurons. With breakdown of the BBB due to the release of proteases including matrix metalloproteinases (MMPs), an influx of immune cells occurs, in turn leading to exacerbation of the initial insult, and formation of vasogenic edema and reactive oxygen species, which further compounds brain injury [145–147]. Although aspects of the inflammatory cascade, particularly in the early phase, can mediate detrimental effects, inflammation in the restoration phase is important to further tissue repair. The release of anti-inflammatory cytokines such as interleukin 10 is crucial in halting the inflammatory process, and thence the release of growth factors by immune cells [145]. Preclinical studies demonstrated that ischemic cells release molecules designated as danger-associated molecular patterns, which act in promoting an inflammatory response via pattern recognition receptors, of which toll-like receptor-4 (TLR-4) is a member. Indeed, mice lacking TLR-4 as well as animals treated with a TLR-4 antagonist have smaller infarct volumes and improved neurological function as compared to wild-type controls [148, 149].

In a mouse middle cerebral artery occlusion (MCAO) stroke model, differential expression of miRNAs in the infarct zone compared to the peri-infarct zone has been observed. This lead to the notion that miRNA regulation of gene expression and/or protein translation in the peri-infarct zone could decrease the inflammatory cascade and improve outcomes. Several groups identified differential miRNA expression showing a proinflammatory array in the infarct zone including miR-181a, and an anti-inflammatory array in the peri-infarct zone including miR-1906, which suppresses TLR-4 and its downstream cascade [148–150]. In the same MCAO model, it was shown that exogenous administration of miR-1906 attenuated the inflammatory cascade, which could be a therapeutic intervention in stroke patients [148–150]. Moreover, *in vitro* studies utilizing the oxygen/glucose deprivation model revealed a therapeutic effect in downregulating the proinflammatory miR-613, which is typically upregulated after an ischemic stroke. The beneficial results were attributed to decreased reactive oxygen species, which relate to lipid peroxidation as well as DNA damage [151]. Lastly, upregulation of miR-21 decreased stroke-related cerebral inflammatory responses thereby increasing BBB integrity, neuronal cell survival, and overall better functional outcomes in a rat model of cerebral ischemia and reperfusion [152]. Preclinical studies reported the efficacy of an herbal extract of Milletia (*Spatholobus suberectus* [DUNN]) to ameliorate oxygen–glucose deprivation-mediated cell death *in vitro* as well as improve histological and biological outcomes in a mouse model of cerebral ischemia. The mechanisms were shown to be related to a decrease in miR-494 levels that led to downstream overexpression of *Sox*8 and activation of the mTOR and MAPK pathway [153]. Recent observations indicated clinical improvement in stroke patients after oral administration of *Spatholobus suberectus* [DUNN] extract [154]. This preliminary data suggests that oral administration of medications targeting miRNA may have the potential to alter gene transcription and expression leading to alteration of the inflammatory response. Randomized trials will be required to confirm these initial observations.

Cerebral Edema

A major clinical challenge is the treatment of inflammation-mediated vasogenic edema, particularly after large hemispheric strokes because of associated high mortality exceeding 70% with maximal conservative management [147]. Currently, hemicraniectomy is the only proven therapy to improve outcomes and reduce mortality; however, even with this highly invasive neurosurgical intervention that requires removal of a large aspect of the skull covering the infarcted hemisphere to relieve swelling, less than 20% of patients will be disability free 1 year after their stroke [155]. Accordingly, novel therapeutic avenues are critically needed to improve outcome in this patient population. In this respect, the water channel aquaporin-4 (AQP-4) and the sulfonylurea receptor 1 (SUR1) and transient receptor potential melastatin 4 (SUR1-

TRPM4) channels have been identified as key targets that promote cerebral edema representing potential targets for early treatment in stroke. Recently, the double-blind, randomized, placebo-controlled phase 2 trial Glyburide Advantage in Malignant Edema and Stroke-Remedy Pharmaceuticals (GAMES-RP) sought to determine whether treatment with the selective SUR1 inhibitor glyburide prevents major disability and death without undergoing decompressive craniectomy in patients with large ischemic infarcts [156]. However, while this trial demonstrated feasibility and safety, there was no difference in the primary outcome between patients receiving glyburide *versus* placebo [156]. Therefore, further study is warranted to assess the potential clinical benefit of a reduction in swelling by targeting these water channels.

AQP-4 acts in 2 distinct patterns depending on the time and cause of edema. AQP-4 enhances early cytotoxic edema by facilitating the transport of water molecules across the cell membrane in astroglial cells. With disruption of the sodium–potassium pump, sodium accumulates in the cells and water molecules traverse the cell membrane through AQP-4 into cells following the concentration gradient. Conversely, AQP-4 has been shown to function in the reabsorption of water molecules from the parenchyma in later-developing vasogenic edema. Hence, inhibiting the AQP-4 water channel is a promising step in decreasing cytotoxic, but more importantly, vasogenic edema [157, 158]. Using an *in vitro* system, siRNAs have been shown to effectively halt AQP-4 translation. In this model, it was shown that water homeostasis was disrupted and downstream gene regulation altered. The silencing of the AQP-4 gene lead to upregulation of *c-fos* as well as nerve growth factor inducible protein-B (NGFI-B), both of which related to apoptosis [159]. Accordingly, utilizing nucleic acid-based approaches may aid our understanding of the precise mechanisms driving cerebral edema formation and thus identify novel therapeutic approaches.

Stroke Recovery

As stated initially, stroke remains a leading cause of disability worldwide [1], which relates to the fact that many patients do not reach the hospital in time to be eligible for acute reperfusion therapies as well as that even those who receive treatment often have significant residual deficits. Therefore, there is a critical need to improve rehabilitative efforts. Restorative therapies that can harness neuroplasticity are a particularly promising strategy because they would be accessible by a large proportion of affected subjects and thus benefit a substantial number of stroke survivors.

While stroke triggers the ischemic cascade leading to tissue injury and inflammation, it also triggers a number of molecular events that aid spontaneous repair via alterations in receptor expression, synaptic and dendritic growth, axonal remodeling, and angiogenesis in the perilesional as well as connected remote brain tissues [160–167]. Hence, there is hope that by modulating these endogenous pathways, such as by small molecules, functional recovery may be improved. Although pharmacological augmentation is important to help manage stroke-related complications such as spasticity, pain, depression, anxiety, and cognitive impairment, almost no strategies exist to truly enhance recovery by pharmacological means. The strongest evidence stems from the Fluoxetine for Motor Recovery After Acute Ischemic Stroke (FLAME) study, which randomized 118 patients 1:1 to receive standard rehabilitative therapies with either placebo or the serotoninergic agent fluoxetine. This trial demonstrated significant improvement in motor function in the fluoxetine group [168], and there are now several phase 3 clinical trials underway to confirm whether the routine administration of fluoxetine after an acute stroke improves patients' functional outcome [169].

A different strategy to improve functional recovery after a stroke is based on observations that ischemic stroke induces widespread changes in gene expression within the neurovascular unit [170, 171]. Thus, there is heightened interest to determine whether it is possible to shift gene expression towards a more proregenerative state such as by modulation of miRNA, which have been shown to be highly expressed in the vasculature, subserve critical vascular cell functions, and their expression profiles are substantially altered in the wake of an ischemic stroke [172–176]. Indeed, several studies indicated that *in vivo* manipulation of cerebral miRNA activity with synthetic miRNA inhibitors and mimics can attenuate ischemic injury and has strengthened the rationale for the development of miRNA-based therapeutic drugs to treat stroke-related brain injury and promote neurological recovery [177–179]. For example, it has recently been shown that axonal alterations of the miR-17-92 cluster expression relate to axonal outgrowth of embryonic cortical neurons [180]. Intravenous injection of mesenchymal stromal cell exosomes containing elevated miR-17-92 cluster into rats 24 h after 2 h of middle cerebral artery occlusion stroke increased neural differentiation, plasticity, and enhanced recovery after stroke [181]. Although infarct volume was not determined in this study, the used stroke model is highly reproducible and typically causes complete infarction within 2 h [182, 183]. Accordingly, one would expect similar infarct volumes at the time of treatment initiation. Consistent with this, poststroke functional deficits prior to treatment were similar between treated and untreated animals as assessed by the modified neurological severity score and foot-fault tests [181]. A further miRNA that has been found significantly altered after brain ischemia is miR-155 [176]. It has been associated with endothelial and vascular function including a role in vascular inflammation, atherogenesis, endothelial cell morphology, and migration, as well as wound healing [179]. Hence, miR-155 may be a suitable target for both modulating postischemic

inflammation and tissue regeneration [179]. Indeed, in a mouse model of focal cerebral ischemia, inhibition of miR-155 by approximately 50% by intravenous injection of an anti-miR-155 miRCURY locked nucleic acid (LNA) inhibitor 48 h after middle cerebral artery occlusion resulted in increased expression of several miR-155 target genes. This improved microvascular perfusion in the peri-infarct brain tissue and reduced vasogenic edema through increased tight junction integrity, which related to less extensive acute infarction as well as delayed neuronal damage and overall improved functional recovery and outcomes [179]. Importantly, the initial infarct volume as assessed by *in vivo* brain MRI showed similar infarct sizes between treated and untreated mice. This is an important observation because the infarct extent is one of the strongest predictors of functional outcome after acute ischemic stroke [184–186]. Together, these studies demonstrate that targeting miRNA may be a novel means to enhance neurorestorative properties of the brain.

Ischemic Stroke Biomarkers

Reliable etiologic classification is critical for effective secondary stroke prevention. However, more than 100 pathological conditions have been implicated in the pathogenesis of ischemic stroke and in a significant proportion of patients, the cause of stroke remains uncertain even after extensive diagnostic evaluation [9, 101, 187, 188]. In addition, many patients have 2 (and more) not mutually exclusive possible stroke causes [101, 187, 188]. In this respect, nucleic acids have been proposed as novel biomarkers that may aid identification of the specific ischemic stroke cause. For example, carotid plaque rupture has been linked to alterations in miRNA expression and several RNAis have been functionally associated with plaque rupture due to thinning of the fibrous cap. The thinning is induced by decreased function and/or levels of miR-221/miR-222, which function in vascular smooth muscle cell proliferation [189]. Similar to these observations, a study including a modestly sized cohort of patients with stable and unstable carotid artery plaques found increased serum miR-221 while circular RNA (circRNA)-284 was elevated in the serum of patients undergoing urgent carotid endarterectomy for symptomatic plaque rupture as compared to asymptomatic patients undergoing the same surgery for a stable plaque. If confirmed, the circRNA-284-to-miR-221 ratio could serve as a noninvasive blood marker for carotid disease [155]. Aside from potentially aiding disease monitoring including the progression of plaque pathology, the development of risk-factor-specific biomarkers could aid clinical decision-making when patients have competing potential stroke mechanisms. Presently, physicians typically resolve to taking a pragmatic approach and treat both conditions. However, this may result in avoidable adverse events such as risk for hemorrhagic complications with a combination of antithrombotic regimens or procedural risk with an intervention of an uncertain level of benefit.

In summary, this article provides an overview of the unique chances and possible advantages of nucleic acid-based therapies as promising future novel treatments across the main phases of ischemic stroke (ranging from stroke prevention to recovery after stroke), and as putative innovative noninvasive blood biomarkers in stroke. Except for antithrombotic and antiatherogenic therapies to stroke prevention, most promising compounds are still within the preclinical discovery and safety stage of development. Considering that the typical drug approval timeline takes approximately 15 years from the earliest stages to clinical approval (~5 years drug discovery, ~2 years for preclinical testing, ~7 years for clinical trials, and ~1 year for approval) [190], substantial additional work remains to be completed before most discussed nucleic acid therapies can be safely implemented in daily practice. We hope that this review will serve as an incentive to forward current scientific efforts in the field of nucleic acid therapy to attenuate the devastating consequences of ischemic stroke.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contributions Dr. Henninger and Dr. Mayasi drafted the article.

Funding Dr. Henninger is supported by K08NS091499 from the National Institute of Neurological Disorders and Stroke of the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Compliance with Ethical Standards

Competing Interests Dr. Henninger serves on the advisory board of Omniox, Inc. and Portola Pharmaceuticals, Inc., and as a consultant for Astrocyte Pharmaceuticals, Inc. Dr. Mayasi declares no competing interests.

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