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Platelet-Associated miRNAs as Potential Diagnostic Biomarkers after Acute Ischemic Stroke

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Für meine Familie

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1 Introduction

1.1 Stroke

Stroke is the second leading cause of death [1] as well as the most common cause of disability and second most common cause of dementia worldwide [2]. Stroke has a tremendous impact on economy, society and healthcare: in the U.S. only, 7 million individuals are affected, which translates into 3% of the entire population. The costs caused by stroke in the U.S. are estimated to be around 60 billion dollars per year [3]. 'Approximately 795,000 strokes occur in the United States each year' [4]. Stroke risk increases remarkably with age: the incidence of stroke in 35- to 44-year-olds is 30-120 in 100,000 per year, while it increases to 670-970 in 100,000 per year in 65- to 74-year-olds. Due to increasing efforts in prevention and awareness of vascular risk factors, incidence rates in highly developed countries have been decreasing over the past decade [5], while they are still increasing in developing countries [6]. With the aging of population especially in Western countries and constant development in low-income countries that results in higher life expectancy, stroke rates are expected to increase drastically over the course of the next decade. It is estimated that in the U.S., a death due to stroke occurs every 4 minutes [7]. The one-month fatality is around 12% for ischemic stroke, while the mortality of hemorrhagic stroke is much higher (33-45%) [8]. Relatively speaking, stroke mortality is higher in men, but in absolute numbers, more women die from a stroke because of the higher life expectancy in women [7].

1.1.1 Risk factors

Risk factors for stroke include demographic factors such as age, sex and heredity [9]. According to the INTERSTROKE study, which involved about 25,000 subjects in 32 countries, cardiovascular diseases, diabetes mellitus, diet and physical activity, and toxic habits such as smoking and alcohol abuse are major factors that contribute to the population-attributable risk [10]. Hypertension is the single most important risk factor in this group [10, 11]. Cardiac diseases, mainly atrial fibrillation and coronary artery disease, are another major risk factor, particularly for ischemic strokes [12]. Diabetes mellitus doubles the risk for an ischemic stroke [13], and it is considered one of the most important risk factors especially because of its atherogenic complications and high prevalence in the population. The same applies for dyslipidemia [11]. Approximately 18% of all strokes can be attributed to active cigarette smoking [11].

1.1.2 Stroke etiology

Approximately 87% of all strokes are ischemic strokes, 10% are hemorrhagic strokes and subarachnoid hemorrhages account for 3% [14]. Ischemic strokes can be characterized as either thrombotic (macroangiopathic or microangiopathic), embolic (with cardiac or arterial origin), or as a result of systemic hypoperfusion (watershed area stroke) [15]. Ischemic strokes can be categorized by the TOAST Classification (Trial of Org 10172 in Acute Stroke Treatment) that was established in 1993 [16]. This classification defines five subtypes: large-artery atherosclerosis, cardioembolism, small-vessel occlusion, other determined etiology or undetermined etiology [16]. Ischemic stroke patients are diagnosed and classified based on brain imaging (computed tomography (CT) and/or magnetic resonance imaging (MRI)), cardiac exams (electrocardiogram (ECG), echocardiography), duplex sonography of extracranial arteries, angiography, and laboratory analyses.
Atherosclerotic changes are the major pathophysiological process that lead to a stroke. Lipid-filled macrophages (so-called foam cells) accumulate in the intima, causing gross changes such as yellowish, fatty streaks in the endothelium of vessels [17]. Lesions may progress to massive extracellular lipid accumulations and complicated fibrous plaques, which are composed of a cap of smooth muscle cells and collagen covering the lipid area in the vessel. Main risk factors for atherosclerosis include hypertension, hypercholesterolemia, cigarette smoking and diabetes mellitus [18]. In an atherosclerotic plaque, several processes lead to an inflammatory response by invasion of monocytes and T-lymphocytes. The plaque can destabilize and rupture, exposing subendothelial collagen which causes platelet activation [19, 20]. Platelet activation can also be caused by shear stress conditions in atherosclerosis. Endothelial injury also leads to activation of the extrinsic pathway of the coagulation cascade, which enhances formation of a thrombus [21]. Thrombus formation can result in a partial or permanent reduction of blood flow by occluding a brain vessel, resulting in a transient ischemic attack (TIA) or an ischemic stroke, respectively [18]. The thrombus can also separate from the primary atherosclerotic site and occlude a smaller downstream vessel, which is defined as an embolic stroke. Most thrombi arise from cerebral arteries or cardiogenic sources [18, 22].

1.1.3 Stroke pathophysiology

In all types of stroke, impaired blood supply of brain tissue causes ischemia and subsequent brain damage: the lack of oxygen and glucose leads to a failure of adenine triphosphate (ATP) production, which then leads to cell injury and death.

Neurons are most susceptible to hypoxia and undergo cell death first, followed by glial cells [15]. The ischemic region can be divided in two parts: the inner core is completely oxygen-deprived, which causes an immediate necrosis of both neurons and glial cells. The outer area, which is called penumbra, is defined as an area with critical hypoperfusion. Cells in this area can possibly be revived by fast intervention and restoration of blood flow. Salvaging the penumbra is the main goal in stroke diagnostics and treatment [23]. An important differential diagnosis of ischemic stroke is the much less common hemorrhagic stroke, which needs to be ruled out prior to starting intravenous thrombolysis treatment, in order to avoid fatal aggravation of the hemorrhage. Hemorrhagic strokes are caused by rupture of a blood vessel, leading to brain hypoxia, parenchyma damage and increased intracranial pressure [24].

1.1.4 The role of platelets in ischemic stroke

Even though platelets are the smallest of all blood cells and only fragments of megakaryocytes, they play a major role in hemostasis, disorders that involve thrombosis, and in ischemic stroke pathophysiology. Platelet production begins with the formation of megakaryocytes and the subsequent fragmentation: In megakaryocyte synthesis, nuclear duplication without cell division results in the formation of giant cells. Cell organelles fuse at specific sites for later nascent platelets within the megakaryocyte, until demarcation by invaginated plasma membranes forms mature platelets [25].

In an intact vessel, platelet activation or coagulation does not occur. Upon activation, platelets change their shape by expressing long dendritic extensions that can facilitate adhesion. Platelets contain two types of granules: The so-called dense granules contain adenosine diphosphate (ADP) and calcium, which support platelet aggregation and platelet-surface coagulation reactions. The α-granules contain numerous proteins such as von-Willebrand-factor and platelet factor 4, which are synthesized in megakaryocytes [26]. Glycoprotein Ib-V-IX, a receptor for von-Willebrand-factor that is located on the platelet membrane and is constantly active, causes platelet attachment to exposed perivascular von-Willebrand-factor [27]. A similar process of platelet attachment occurs when exposed collagen from subendothelial matrix binds glycoprotein Ia-IIa. Glycoprotein IIb-IIIa, the most abundantly expressed surface protein on platelets, changes conformation during platelet activation in order to allow it to bind fibrinogen, a process that mediates platelet aggregation [28].

Various cerebrovascular and cardiovascular events can be traced back to atherosclerosis, which may lead to plaque rupture and thrombosis [29]. Rupture of an atheromatous plaque induces several processes such as platelet adhesion, activation and aggregation. Platelet aggregation and formation of a blood clot causes vessel occlusion or distal embolization, which is a critical event in the pathophysiology of stroke, myocardial infarction and acute ischemia of extremities [30]. Since the role of platelet aggregation in those diseases has been determined, antiplatelet drugs have emerged as a key approach in secondary prevention of serious vascular events [31].

ASA, or acetylsalicylic acid, is the most frequently prescribed antiplatelet drug for patients with severe atherosclerosis and risk factors for vascular events. ASA inhibits thromboxane A2 production by inhibiting cyclooxygenase, the key enzyme in thromboxane A2 synthesis [32].

1.1.5 Stroke symptoms

Stroke usually presents with sudden onset of symptoms caused by acute occlusion of a brain vessel and subsequent brain tissue hypoxia. Symptoms vary remarkably depending on severity and location of the vessel occlusion: possible signs are deficiencies in consciousness, orientation, pupillary response and motor activation, ataxia, paralysis, paresthesia, loss of reflexes, neglect or dysarthria [33].

1.1.6 Stroke diagnostics

Neuronal damage progresses with every minute of ischemia: it has been estimated that every minute, 1.8 million neurons are lost irreversibly [34]. Thus, a quick diagnosis and early intervention are crucial in the acute phase of stroke.

Cranial imaging is the single most important measure to quickly examine a patient presenting with stroke symptoms. It is crucial to rule out an intracranial hemorrhage, which cannot be distinguished from an ischemic stroke based on clinical symptoms only, in order to proceed to lysis or recanalization treatment as fast as possible. Both CT and MRI are used in the emergency department for diagnosis but show significant advantages and disadvantages in acute stroke diagnostics: CT scans are most commonly used in patients with suspected stroke. They are fast, easily available, and less expensive than an MRI, and are thus widely used in the acute phase of a stroke. They are also less susceptible to motion artifacts and available for all patients. While showing high sensitivity for

intracranial hemorrhage, the CT lacks sensitivity for detecting an acute ischemic stroke in the first hours of onset [34]. These can be detected much better with MRI imaging [35]. However, patients with intracorporal devices such as pacemakers are not eligible for an MRI.

The second goal in stroke diagnostics, after ruling out intracranial hemorrhage, is to define infarct core and penumbra in order to determine how much brain tissue could be salvaged by early thrombolysis therapy or mechanical thrombectomy [36]. Discriminating the penumbra requires a CT- or MR-based perfusion imaging: in both scans, intravenous contrast medium is applied, and cerebral blood flow and cerebral blood volume are measured. The infarct core, which contains irreversibly damaged brain tissue, can be distinguished from the penumbra, which is defined as an area without perfusion, but with remaining diffusion, which suggests viable brain tissue that could regain normal function when blood flow is restored rapidly enough [37].

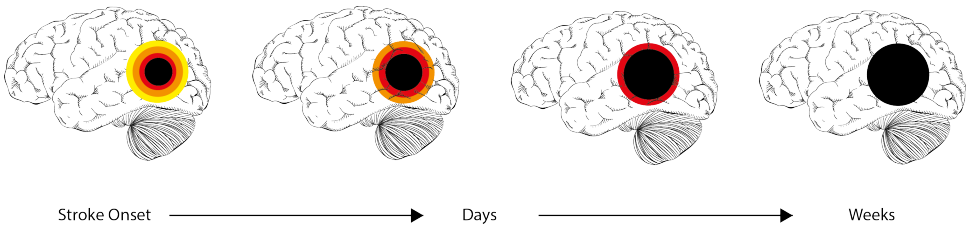

Figure 1: Evolution of penumbra and infarct core
When blood flow to a brain area is impaired, the infarct core defines the area of irreversible neuron damage. The surrounding area, the so-called penumbra, includes viable brain tissue in an area with no perfusion, in which neurons could possibly be salvaged by restoration of blood flow. Without treatment, neurons in the penumbra undergo apoptosis and the infarct core takes over the penumbra area over the course of weeks. Figure adapted from Dirnagl et al., 1999 [38].

1.1.7 Stroke therapy

Early intervention such as reopening of the artery and restoration of blood flow can salvage ischemic brain tissue and limit infarct consequences. Time passed before achieving recanalization, size of the ischemic area and collaterals are major determinants for outcome and life expectancy. Nowadays, the most commonly used treatment option is intravenous tissue plasminogen activator (tPA), which causes rapid intraarterial thrombus dissolution. It has been shown that patients who receive tPA treatment are 30% more likely to have minimal or no residual disabilities after 3 months compared to placebo controls [39]. The time frame for tPA administration is considered 4.5h after symptom onset [40], however, recent

studies revealed that patients who show salvageable brain tissue in perfusion imaging benefit from thrombolysis therapy up to 9 hours after symptom onset [41]. Patients with unknown onset of symptoms (e.g. wake-up stroke) have been shown to benefit from thrombolysis beyond the 4.5-hour time frame, if a mismatch between diffusion-weighted imaging and FLAIR (fluid-attenuated inversion recovery) sequence can be detected [42].

In addition to intravenous thrombolysis, endovascular recanalization has emerged as an important treatment option for ischemic stroke patients with an occlusion of a brain vessel in the proximal anterior circulation within the first 6 hours after symptom onset [43]. A recent study showed that patients might even benefit from mechanical thrombectomy up to 24 hours after symptom onset, if they present with an infarct volume and clinical symptom severity mismatch [44].

1.2 Transient ischemic attack (TIA)

A transient ischemic attack (TIA) is characterized by sudden onset of neurological symptoms that completely resolve without intervention. According to the WHO criteria proposed in 1988, a TIA used to be defined as symptoms resolving in < 24 hours [45], under the assumption that no permanent brain damage occurs in a TIA. However, since the introduction of more accurate diagnostic means such as high-resolution diffusion-weighted imaging (DWI), it has been suggested that a considerable number of TIA patients do, in fact, show signs of permanent tissue damage in brain imaging [46]. This led to a redefinition of a TIA that is independent of time: 'A transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia, without acute infarction' [47]. There are current attempts to further redefine TIA and stroke, since it has recently been shown that the majority of TIA patients show evidence of brain injury [48]. A TIA is the most important risk factor for an ischemic stroke, the risk for which is estimated to be 12-20% in the first three months after a TIA or a minor stroke [49]. Diagnostic measures, primary and secondary prevention for ischemic stroke equally apply for TIA patients. Even though 10% of minor stroke and TIA patients show an intracranial occlusion in neurovascular imaging [50] thrombolysis treatment is not eligible for those patients [51]. Among patients with acute deficits that show no signs of tissue infarction upon admission, a biomarker that could distinguish those patients from both ischemic stroke patients and patients with other neurological pathologies would a remarkable improvement in diagnostic accuracy and efficiency in emergency treatment compared to neuroimaging, as well as in choosing the best treatment option for the patient.

1.3 Stroke biomarkers

Currently, cranial imaging is the most useful diagnostic measure to assess stroke patients with acute neurological symptoms. It can rule out other pathologies that present with similar symptoms and determine the treatment course upon hospital arrival. However, the disadvantages of CT and MRI have led to the desire of finding a biomarker that could support imaging in acute stroke diagnostics. An example of successful usage of blood-based biomarkers in the clinical emergency setting can be observed in the identification of cardiac isoenzymes troponin I and T, which has revolutionized diagnostics of acute myocardial infarction [52].

A diagnostic biomarker for ischemic stroke requires high sensitivity and should have high specificity in order to distinguish ischemic stroke patients from healthy controls, patients with a TIA or hemorrhagic stroke as well as from patients with stroke mimics such as seizures, migraine or cerebral infections. Ideally, it should also be able to distinguish stroke patients from other ischemic pathologies such as myocardial infarctions or pulmonary embolisms. However, a diagnostic biomarker could lack specificity and still be useful in the clinical setting by ruling out a disease (such as D-Dimer test in pulmonary embolism) [53], or support diagnosis in diseases with similar pathophysiological processes but entirely different clinical presentation, such as myocardial and cerebral infarctions.

Difficulties in the search for blood-based biomarkers for stroke include delayed rise of plasma levels of brain-derived biomarkers in the blood stream due to the blood-brain barrier. Some potential biomarkers show correlation to infarct size, however, even a small infarction in an important area of the brain, such as Broca's area, could cause remarkable disability that cannot be quantified by a biomarker [54].

Also, techniques to quickly and accurately measure levels of the biomarker need to be widely available. So far, multiple studies have tried to identify blood- or cerebrospinal fluid (CSF)-based biomarkers, but none of them fulfilled all of these requirements [55].

1.3.1 Neuronal and glial markers

S-100-B (S100B) is a calcium-binding peptide, which is secreted by astrocytes after brain injury or neurodegenerative processes and has been suggested to exert neuroprotective actions [56]. Increased serum levels have been observed after ischemic stroke, but also after brain trauma or in the setting of Alzheimer's

disease or psychiatric diseases [57]. S100B levels have been shown to correlate with infarction size and to be associated with higher risk for hemorrhagic transformation [58].

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein, which is part of the cytoskeleton of astrocytes and ependymal cells. Increased serum levels are specific for cerebral injury [59]. GFAP has been shown to be more sensitive than S100B, particularly in smaller lesions and minor strokes. However, both S100B and GFAP show delayed rise and peaks of serum concentration levels after ischemic stroke, which limits their usefulness as a diagnostic biomarker in the acute phase [60, 61].

Increased neuron-specific enolase (NSE) levels in the CSF occur after ischemic stroke, but also after traumatic brain injuries or intracerebral bleeds [62]. Levels peak at 4-8 hours after symptom onset, but serum and CSF levels differ widely and do not seem to show a consistent change after ischemic stroke [63].

Neurofilaments are part of the structural scaffold of neurons. They consist of three subunits, with the neurofilament light protein (NfL) being the core of the filament. A recent study of our group showed that ischemic stroke patients have higher serum NfL levels on the day of stroke onset compared to a healthy control group, with a peak at 7 days. Furthermore, NfL levels were shown to correlate with infarct volumes, and to predict clinical outcome [64]. However, diagnostic utility in the setting of an acute stroke was inferior to miR-125a-5p, miR-125b-5p and miR-143-3p that we previously identified as biomarkers of acute ischemic stroke [65]. Also, elevated NfL serum levels in a patient with suspected acute ischemic stroke could possibly be caused by previous minor ischemic events weeks or months prior to current symptom onset, since it has been shown that serum NfL levels remain elevated for months after a stroke event [64].

1.3.2 Coagulation markers

Since thrombus formation is an essential part in ischemic stroke pathophysiology, coagulation markers might show potential as diagnostic biomarkers: Thrombomodulin is a protein that is present in the endothelial membrane. It binds thrombin and activates protein C and is therefore part of an important antithrombotic mechanism in the physiological state. Protein expression in the brain is controlled by astrocytes [66]. Some types of thrombomodulin have been

shown to be associated with cardiovascular events, but not with stroke events [67], and do not show significant changes in stroke patients compared to control groups [68].

Since other unspecific coagulation markers such as D-Dimers have been shown to be very useful as diagnostic biomarkers in other diseases [53], recent studies addressed other coagulation markers that could possibly be associated with ischemic stroke:

Increased fibrinogen levels after ischemic stroke have been shown to correlate with infarction size and outcome [69, 70]. Also, fibrinopeptide A, β-thromboglobulin, prothrombin fragments 1 and 2, thrombin-anti-thrombin complexes, platelet factor 4 and von-Willebrand-factor plasma levels have been shown to be associated with stroke severity [71, 72]. D-Dimers have been shown to be acutely elevated in ischemic stroke [73], but are not sensitive enough to serve as a diagnostic biomarker for ischemic stroke [74].

1.3.3 Inflammatory markers

After stroke, a local immune response is always present in the ischemic area of the brain: Cytokines are released in order to mediate inflammatory and immune responses [75]. Neuronal and glial cells actively and passively secrete interleukins, transforming growth factor beta (TGF-β), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), selectins or tumor-necrose factor alpha (TNFα) into the blood stream [76]. C-reactive protein (CRP) is produced in the liver in response to interleukin-6 (IL-6) activation and has a crucial role in inflammatory processes [77]. Since atherosclerosis occurs based on inflammatory reactions in the endothelium, increased CRP levels have been shown to be associated with higher risk for stroke [78], infarct volume, stroke severity, clinical outcome [79], and risk for recurrent stroke [80]. However, none of those markers of systemic or local immune response have proven to have neither the sensitivity nor specificity that a diagnostic biomarker for ischemic stroke requires.

So far, most serum-based biomarkers showed poor results in prospective testing in acute diagnosis of ischemic stroke overall [60], mostly because their distribution patterns overlap considerably in patient and healthy control groups. Another difficulty is that different population groups may show different distribution and cut-off levels [81, 82]. Also, many biomarkers show a delayed peak days after

the initial event, which excludes them from being useful in an emergency department setting [60, 62]. In most cases, other neurologic diseases such as migraine, seizures, infections and dementia can present as stroke mimics and cause alterations in biomarker levels as well [83]. So far, none of the studies investigating biomarkers has shown results, which would be promising to facilitate ischemic stroke diagnostics or replace cranial imaging in ischemic stroke diagnostics in the near future.

1.4 MicroRNAs

'microRNAs (miRNAs) are endogenous, $\sim$22 nucleotide RNAs that can play important roles in animals and plants by targeting mRNAs for cleavage or translational repression' [84]. They were first discovered in 1993 [85]. Ever since their discovery, knowledge about their biogenesis and functions has grown due to their potential for diagnostic and therapeutic use. Nowadays, there are more than 2500 different miRNAs identified in humans, with numbers constantly increasing [86].

1.4.1 Biosynthesis

miRNA genes are widely spread over the entire genome. One gene can either encode for a single miRNA hairpin precursor or for multiple precursors. The primary miRNAs (pri-miRNAs) 'act as substrates for two members of the RNase III family of enzymes, Drosha and Dicer. The product of Drosha cleavage, an $\sim$70 nucleotide pre-miRNA [(precursor miRNA)] , is exported to the cytoplasm where Dicer processes it to an $\sim$20 base pair miRNA/miRNA* duplex' [87]. The strands are named 5p and 3p. The mature single strands then join the RNA-induced silencing complex (RISC), which can now bind the 3'-untranslated region (3'-UTR) of mRNAs with their 5'-untranslated region (5'-UTR) [87].

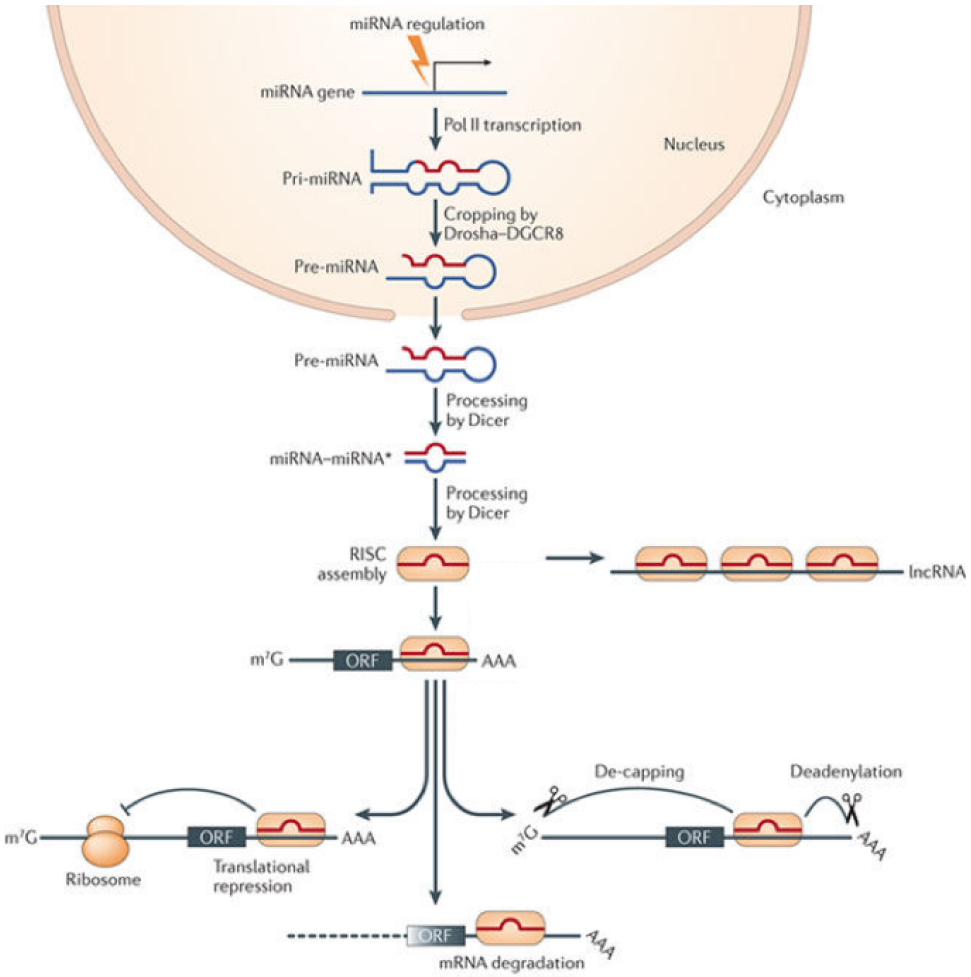

Figure 2: miRNA biogenesis and mechanisms of action
Transcription of miRNAs as described above. In most cases, only one of the strands is incorporated into the RNA-induced silencing complex (RISC). The RISC complex can recognize target regions on messenger RNAs (mRNAs). Figure from van Rooij, Olson 2012 [88]. Permission granted by Springer Nature.

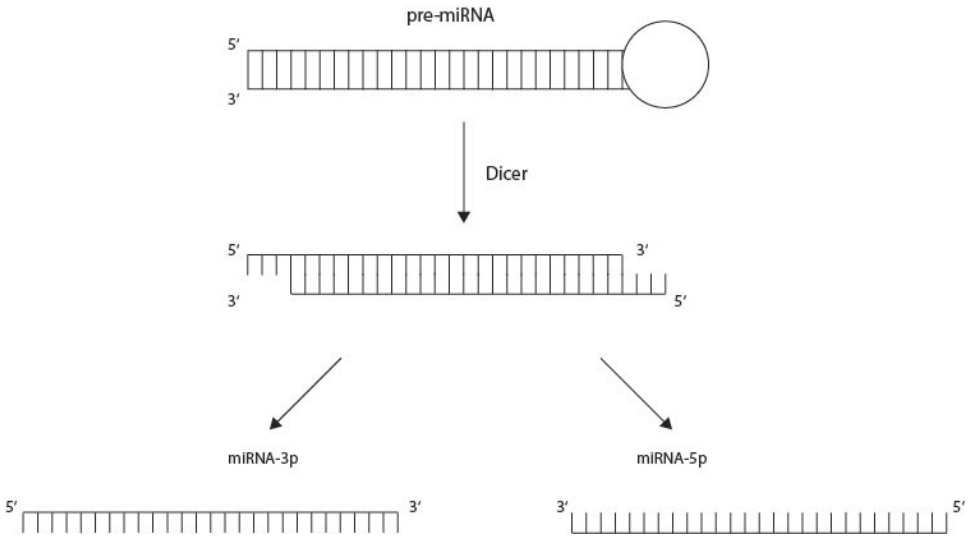

Figure 3: Biogenesis of miRNAs
Two miRNAs derive from the same precursor, the so-called pre-miRNA. Since both resulting miRNAs are only partially complementary, they can possess remarkably different functions and be involved in different pathways. Figure modified from Schober et. al., 2014 [89].

1.4.2 Functions

The RISC can downregulate gene expression by post-transcriptional regulation of messenger RNAs (mRNAs): if the mRNA has sufficient complementarity to the miRNA incorporated in the RISC, the target mRNA can be cleaved by the complex. In case of a short area of mRNA-miRNA complementarity, the RISC can bind the 3'-UTR of the target mRNA, thereby repressing translation [84]. Usually, the miRNA-mRNA complementary base-pairing is only 6-8 base pairs, which allows the miRNA to bind different mRNAs [87]. Even though the exact regulatory mechanisms are still unknown, many miRNAs have been shown to be involved in neuronal gene expression [90], brain morphogenesis [91] and stem cell division [92].

1.4.3 Circulating miRNAs

Beyond their intracellular function, some miRNAs are present in blood plasma. They 'are remarkably stable in plasma and serum, and resistant to RNase activity, as well as extreme pH and multiple numbers of freeze-thaw cycles' [93]. While endogenous miRNAs remain stable under experimental conditions, synthetic miRNAs that are added to plasma are quickly degraded by RNAses [94], suggesting a protective mechanism to prevent degradation of endogenous miRNAs. 'miRNAs are carried by encapsulation in membrane-derived vesicles such as exosomes, microvesicles, apoptotic bodies, RNA-binding proteins such

as Argonaute 2, or lipoprotein complexes such as HDL' [95].
reduThe discovery of miRNAs that are present in microparticles has also raised the question as to whether miRNAs are actively secreted by a cell, taken up by a different target cell, potentially regulating its gene expression and thus participating in cell-to cell communication [96, 97].

The remarkable stability of circulating miRNAs, their characteristic expression patterns in different tissue or cell types, and the fact that miRNA levels can easily be detected and quantified with high sensitivity and specificity by real-time polymerase chain reaction (PCR) and microarrays [98] has led to the initiation of multiple studies that focus on their use as biomarkers in diagnostics or predicting outcome, for example in cardiovascular diseases or diabetes mellitus [99]. For example, four cardiac miRNAs (miR-1, miR-133, miR-208a and miR-499) have been found to show an elevation in plasma levels of patients in the acute phase of myocardial infarction [100, 101].

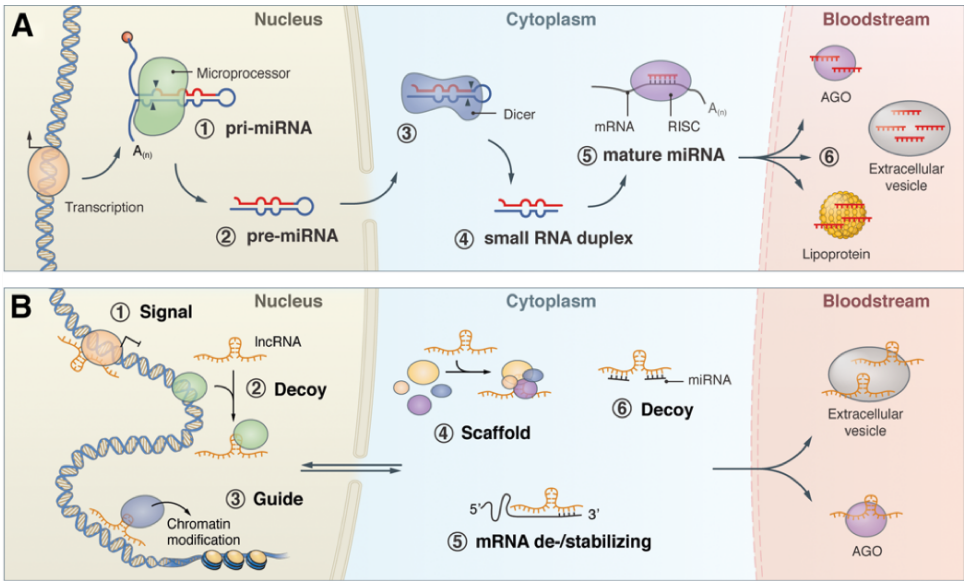

Figure 4: Biogenesis of miRNAs and functions of long non-coding RNAs
A: miRNA biogenesis in nucleus and cytoplasm. Mature miRNAs are transported by vesicles or proteins, which prevent them from degradation. B: Functions of long non-coding RNAs. Image by Dr. Johannes Richers. AGO = Argonaute protein; pre-miRNA = precursor microRNA; pri-miRNA = primary microRNA; RISC = RNA-induced silencing complex. Figure from Tiedt, Dichgans 2018 [102]. Permission granted by Wolters Kluwer Health, Inc.

1.4.4 Platelet-associated miRNAs

Platelet aggregation is an essential pathophysiological process in both embolic and thrombotic ischemic stroke. Upon activation, platelets release microparticles that contain proteins and inflammatory mediators, but also circulating miRNAs

that use microparticles as a protective medium and as a transport vehicle in plasma [103]. It is assumed that platelets inherit their miRNA content from megakaryocytes [104], and for many circulating miRNAs, platelets have been identified as a major cellular source [105]. It has been discovered that megakaryocytes strongly contribute to circulating miR-126 levels [106], a well-studied miRNA that has been reported to be specifically expressed in endothelial cells when first identified [107].

Up to now, about 750 miRNAs have been identified as being associated with platelets [108]. Platelet miRNA plasma levels in healthy individuals remain stable over the lifespan of a platelet [109]. 'In response to antiplatelet drugs, circulating levels of many miRNAs are reduced in platelet-poor plasma (PPP)' [108]. Levels can also be altered in various diseases, such as myocardial infarction [110], diabetes mellitus [111], and cancer [112].

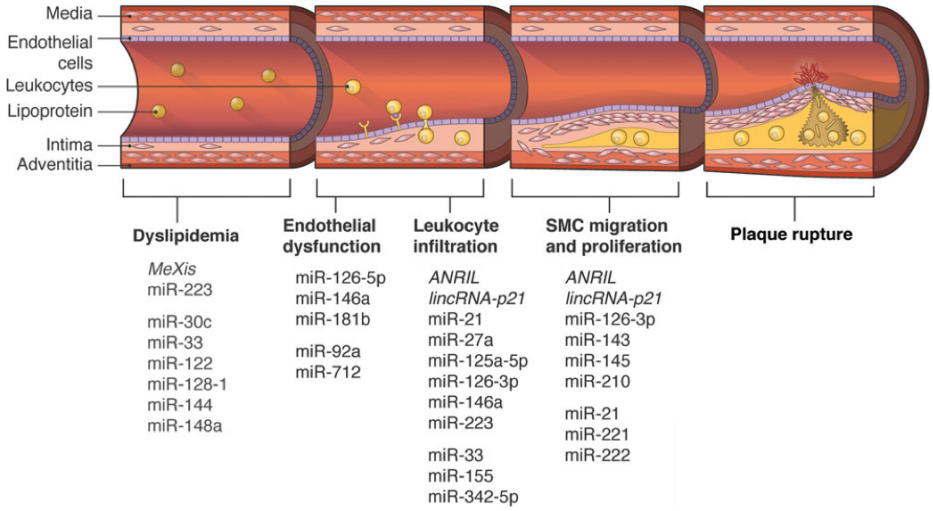

Figure 5: Non-coding RNAs regulating atherogenesis
Association of long non-coding RNAs and miRNAs to different stages of atherosclerosis. Image by Dr. Johannes Richers. SMC = smooth muscle cell. Figure from Tiedt, Dichgans 2018 [102]. Permission granted by Wolters Kluwer Health, Inc.

1.5 Prior research in our group

Our group has been interested in identifying miRNAs that show altered expression patterns in acute ischemic stroke patients. By RNA sequencing, we identified differentially expressed circulating small, non-coding RNAs in the acute phase of ischemic stroke and identified, validated and replicated three miRNAs, that were upregulated in the acute phase of ischemic stroke and transient ischemic attack patients compared to healthy controls: miR-125a-5p, miR-125b-5p and miR-143-

3p [65]. A random forest classification showed an area under the curve (AUC) of 0.90 for the combination of all three miRNAs, which was superior to cranial CT and previously reported biomarkers such as NSE or IL-6. Longitudinal analysis of circulating levels of those three miRNAs up to 90 days after stroke showed normalization in expression levels of miR-125b-5p and miR-143-3p on day 2 after stroke, while miRNA-125a-5p levels remained elevated over the course of 90 days. 'Levels of miR-125a-5p, miR-125b-5p, and miR-143-3p were not affected by chemical hypoxia of Neuro2a cells in vitro and in 2 different experimental stroke models' [65]. 'All 3 microRNAs depended on platelet numbers in a platelet spike-in experiment' [65].

2 Objectives and Goal of the Dissertation

Since stroke is one of the leading causes of death and disability worldwide, requires fast diagnosis and treatment in order to minimize permanent brain damage and disability, a fast, widely available diagnostic tool with both high sensitivity and specificity would be of vital importance. As reviewed before, no diagnostic measure that meets all of these criteria has been identified yet. So far, neuroimaging is the best and most accurate way to diagnose a stroke. CT scans can rule out intracranial hemorrhage with high sensitivity, but lack sensitivity in distinguishing ischemic stroke from stroke mimics such as seizures, migraines or cerebral infections early after symptom onset. MRI imaging has high sensitivity in the early stages of stroke but is often considered to be too time-consuming to be used in a critical clinical situation.

An ideal biomarker for ischemic stroke should have high sensitivity as well as high specificity, in order to quickly diagnose an ischemic stroke and to rule out a TIA or a stroke mimic. A blood-based biomarker should present with rising plasma levels in the early hours after symptom onset and make it possible to distinguish ischemic stroke patients from healthy people and from people with other pathologies. It should be cost-efficient, easily and quickly measurable in the emergency department setting, as well as valid and reliable. So far, all previously suggested blood-based biomarkers such as NSE, GFAP or S100B have failed to meet those criteria. miRNAs circulate in the blood with high stability and show tissue-specific expression patterns, thus showing potential as biomarkers for disease states. Since platelet function, activation and aggregation is a major part of ischemic stroke pathophysiology, we here investigated the diagnostic value of platelet-associated miRNAs.

The goal of this project is 1) to identify miRNAs that show differential plasma levels in ischemic stroke patients compared to TIA patients and healthy subjects 2) to assess the diagnostic value of these miRNAs in a clinical setting and 3) to analyze miRNA levels in murine stroke in a first step towards investigating molecular mechanisms of miRNA production, release, circulation and regulation in ischemic stroke pathophysiology.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

-20℃ Freezer	Co. Liebherr (Bulle, CH)
-80℃ Freezer	Co. Thermo Fisher (Waltham, MA, USA)
4℃ Freezer	Co. Siemens (Munich, D)
Accu-jet Pipette Controller	Co. BrandTech (Essex, CT, USA)
autoMACS Pro Cell Separator	Co. Miltenyi Biotec (Bergisch-Gladbach, D)
Centrifuge 5427 R	Co. Eppendorf (Hamburg, D)
Centrifuge 5810 R	Co. Eppendorf (Hamburg, D)
Centrifuge Heraeus Megafuge 16	Co. Thermo Fisher (Waltham, MA, USA)
Centrifuge Peqlab Perfect Spin 24 R	Co. VWR Intern. (Center Valley, PA, USA)
Confocal Microscope Sp5	Co. Leica (Wetzlar, D)
CryoStat NX70	Co. Thermo Fisher (Waltham, MA, USA)
Gilson Pipettes (10µl, 20µl, 200µl, 1000µl)	Co. Thermo Fisher (Waltham, MA, USA)
Heater Thermocenter	Co. SalvisLab (Rotkreuz, CH)
Hellendahl Glass Box	Co. Thermo Fisher (Waltham, MA, USA)
Ice Machine	Co. Ziegra (Isernhagen, D)
Microscale Balance	Co. Acculab (Central Islip, NY, USA)
Microtome CryoStat NX70	Co. Thermo Fisher (Waltham, MA, USA)
Millipore Water Suspenser	Co. Merck (Darmstadt, D)
Multi-Channel Pipet 10µl, 100µl	Co. Eppendorf (Hamburg, D)
Multipette	Co. Eppendorf (Hamburg, D)
Nitrogen Cryo Tank	Co. Cryo Anlagenbau (Wilnsdorf, D)
Pap Pen	Co. Kisker Biotech (Steinfurt, D)
pH-Meter	Co. SI Analytics (Rye Brook, NY, USA)
Precision Balance	Co. Acculab (Central Islip, NY, USA)
Roche LightCycler 480/II	Co. Roche (Basel, CH)
ThermoMixer Pro	Co. CellMedia (Elsteraue, D)
Veriti Thermal Cycler	Co. Thermo Fisher (Waltham, MA, USA)
Vortex Shaker Peqlab	Co. VWR Intern. (Center Valley, PA, USA)

3.1.2 Consumables

396-well Light Cycler qPCR Plate	Co. Roche (Basel, CH)
96-well MicroPlate	Co. Sigma-Aldrich (St. Louis, MO, USA)
Butterfly Venofix	Co. Braun (Melsungen, D)
Combitips 2ml, 5ml	Co. Eppendorf (Hamburg, D)
Costar Stripettes 5ml, 10ml, 20ml	Co. Sigma-Aldrich (St. Louis, MO, USA)
DNA LoBind Tubes 0.5ml	Co. Eppendorf (Hamburg, D)
DNA LoBind Tubes 5ml	Co. Eppendorf (Hamburg, D)
EDTA 9ml Monovette	Co. Sarstedt (Hildesheim, D)
FACS Polystyrene Test Tubes	Co. Elkay (Basingstoke, UK)
Falcon 50ml Tubes	Co. Thermo Fisher (Waltham, MA, USA)
Filter Tips 10µl, 20µl, 200µl, 1250µl	Co. VWR Intern. (Center Valley, PA, USA)
Fluoromount Mounting Medium	Co. Sigma-Aldrich (St. Louis, MO, USA)
Gloves Vasco Powder-Free	Co. Braun (Melsungen, D)
Kimwipes KimTech	Co. Thermo Fisher (Waltham, MA, USA)
Menzel Cover Glasses 24x60mm	Co. VWR Intern. (Center Valley, PA, USA)
Microvette 300µl	Co. Sarstedt (Hildesheim, D)
Parafilm	Co. Sigma-Aldrich (St. Louis, MO, USA)
Pipet Tips TipOne	Co. Starlab (Hamburg, D)
RNAse Away Spray	Co. Thermo Fisher (Waltham, MA, USA)
Sealing foil 386-well Plate	Co. Roche (Basel, CH)
Sealing foil 96-well Plate	Co. Sigma-Aldrich (St. Louis, MO, USA)
Superfrost Plus Slides	Co. Thermo Fisher (Waltham, MA, USA)
Tissue Tek O.C.T. Compound	Co. Scigen (Gardena, CA, USA)
VWR Reagent Reservoir	Co. VWR Intern. (Center Valley, PA, USA)

3.1.3 Chemicals

0,5M EDTA, pH 8.0	Co. Invitrogen (Carlsbad, CA, USA)
1-Methylimidazole	Co. Sigma-Aldrich (St. Louis, MO, USA)
12M HCl	Co. Carl Roth (Karlsruhe, D)
Bovine serum albumin (BSA)	Co. Sigma-Aldrich (St. Louis, MO, USA)
Citric Acid	Co. Sigma-Aldrich (St. Louis, MO, USA)
Deionized formamide	Co. Life Technologies (Carlsbad, CA, USA)
Dextran sulfate	Co. Sigma-Aldrich (St. Louis, MO, USA)
EDC(N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride)	Co. Sigma-Aldrich (St. Louis, MO, USA)
Ethanol Absolute for Analysis	Co. Sigma-Aldrich (St. Louis, MO, USA)
Ficoll 400 PM	Co. Sigma-Aldrich (St. Louis, MO, USA)
Glycine	Co. Sigma-Aldrich (St. Louis, MO, USA)
Glycogen Ambion	Co. Thermo Fisher (Waltham, MA, USA)
HISTOPAQUES 1077	Co. Sigma-Aldrich (St. Louis, MO, USA)
Isopropanol	Co. Sigma-Aldrich (St. Louis, MO, USA)
MACS Running Buffer	Co. Miltenyi-Biotec (Bergisch-Gladbach, D)
MACS Washing Buffer	Co. Miltenyi-Biotec (Bergisch-Gladbach, D)
NaCl	Co. Carl Roth (Karlsruhe, D)
Normal goat serum (NGS)	Co. Sigma-Aldrich (St. Louis, MO, USA)
Nuclease-free water	Co. Qiagen (Venlo, N)
Polyvinylpyrrolidone	Co. Sigma-Aldrich (St. Louis, MO, USA)
Potassium dihydrogen phosphate	Co. Sigma-Aldrich (St. Louis, MO, USA)
Rotihistol (Xylene)	Co. Carl Roth (Karlsruhe, D)
Salmon sperm DNA	Co. Applichem (Gatersleben, D)
Sodium Chloride	Co. Sigma-Aldrich (St. Louis, MO, USA)
Sodium Citrate	Co. Sigma-Aldrich (St. Louis, MO, USA)
Sodium phosphate dibasic dihydrate	Co. Sigma-Aldrich (St. Louis, MO, USA)
TRIS	Co. Sigma-Aldrich (St. Louis, MO, USA)
Tween 20%	Co. Carl Roth (Karlsruhe, D)
UltraPure 10% SDS solution	Co. Invitrogen (Carlsbad, CA, USA)
UltraPure 20x saline sodium citrate (SSC)	Co. Invitrogen (Carlsbad, CA, USA)
Yeast tRNA	Co. Invitrogen (Carlsbad, CA, USA)

3.1.4 Buffers and solutions

Platelet spike-in experiment

Phosphate-buffered saline (PBS)	800ml Millipore Water 8g NaCl 0.2g KCl 1.44g Na_2PO_4 0.24g KH_2PO_4

Fluorescent in-situ hybridization (FISH)

DEPC-H_2O	1ml Diethylpyrocarbonate (DEPC) 1l Millipore Water (0,1% v/v)
70% Ethanol	700ml 100% Ethanol 300ml DEPC-H_2O
50% Ethanol	500ml 100% Ethanol 500ml DEPC-H_2O
NaCl (3M)	87.66g NaCl 500ml DEPC-H_2O
Tris-HCl (1M, pH 7.4/8.0)	121.1g Tris base 800ml DEPC-H_2O Adjust to pH 7.4 or 8.0 by adding 12M HCl Fill up to 1l with DEPC-H_2O
10x TBS (Tris-buffered saline)	500ml 1M Tris-HCl pH 7.4 90g NaCl 410ml DEPC-H_2O
Sodium Citrate 0.1M	29.41g Sodium citrate 1l DEPC-H_2O

50x Denhardt's solution	1g Ficoll 400 150ml DEPC-H_2O 1g BSA (bovine serum albumin) 1g Polyvinylpyrrolidone
Hybridization buffer	1ml Tris-HCl pH 8.0 50ml deionized formamide 2.5ml 10% SDS 20mg yeast tRNA (final concentration 500µg/ml) 2ml 50x Denhardt's solution (final concentration 1x) 20ml 3M NaCl (final concentration 600mM) 2.5ml 0.5M EDTA 10g Dextrane sulfate (final concentration 10%) 100ml DEPC-H_2O
Diluted SSC	1x: 50ml 20x SSC + 95ml DEPC-H_2O 2x: 10ml 20x SSC + 90ml DEPC-H_2O 0.2x: 1ml 20x SSC + 99ml DEPC-H_2O
Citric Acid 0.1M	9.56g Citric acid 500ml DEPC-H_2O
Methylimidazole buffer	1.6ml 1-Methylimidazole 130ml DEPC-H_2O Adjust to pH 8.0 with 12M HCl 16ml NaCl 3M 12ml DEPC-H_2O
EDC solution	10ml Methylimidazole buffer 307mg EDC
Blocking buffer	0.1g BSA (bovine serum albumin) 5ml PBS 0.3g NGS (normal goat serum)

10x PBS	800g NaCl 20g KCl 144g $Na_2HPO_4^{2-}$ + $2H_2O$ 24g KH_2PO_4

3.1.5 Primers, probes and kits

Platelet spike-in experiment

CD61 MicroBeads human	Co. Miltenyi Biotec (Bergisch-Gladbach, D)

RNA isolation

miRCURY RNA-Isolation Kit for Biofluids	Co. Exiqon (Vedbaek, DK)
RNA Spike-in Kit, Uni-RT	Co. Exiqon (Vedbaek, DK)

cDNA synthesis

Universal cDNA Synthesis Kit II	Co. Exiqon (Vedbaek, DK)
Uni-Sp6 LNA PCR Primer Set	Co. Exiqon (Vedbaek, DK)

RT-qPCR (reverse transcription quantitative polymerase chain reaction)

Exilent SYBR Green Master Mix	Co. Exiqon (Vedbaek, DK)

Fluorescent in-situ hybridization (FISH)

FISH LNA probes, 5'- and 3' DIG-labeled	Co. Exiqon (Vedbaek, DK)
FISH Scramble probes, 5'- and 3' DIG-labeled	Co. Exiqon (Vedbaek, DK)
α-NeuN mouse	Co. Abcam (Cambridge, UK)
α-GFAP mouse	Co. Abcam (Cambridge, UK)
α-digoxigenin POD	Co. Sigma-Aldrich (St. Louis, MO, USA)
Anti-mouse Alexa Flour 488	Co. Abcam (Cambridge, UK)
Cy3 TSA	Co. Perkin Elmer (Waltham, MA, USA)
DAPI	Co. Thermo Fisher (Waltham, MA, USA)

3.1.6 miRNAs

microRNA	Sequence (5' –> 3')
hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG
hsa-miR-21-5p	UGCUUAUCAGACUGAUGUUGA
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-26a-3p	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA

Table 1: Sequences of miRNAs used in the experiments

3.2 Methods

3.2.1 Patient recruitment and study population

During a three-year period from 2014 to 2017, ischemic stroke patients and transient ischemic attack patients were recruited through the emergency department at the Klinikum der Universität München, the university hospital at Ludwig-Maximilians-Universität, Munich. Healthy control subjects were recruited through the outpatient clinic of the Institute for Stroke and Dementia Research, and were matched for age, sex and vascular risk factors. We included patients over the age of 18, with less than 24 hours between symptom onset and blood draw upon hospital arrival. Only patients who later had a final diagnosis of ischemic stroke, which was defined by an acute focal neurologic deficit as well as evidence for stroke on neuroimaging (positive lesion in diffusion-weighted imaging on MRI, or a new lesion on a delayed CT scan), were included in the stroke group. For the derivation sample, we excluded patients with active malignant, inflammatory or infectious diseases or surgery within the last month. We also excluded patients who were treated with low-molecular or unfractionated heparin, patients with intake of antiplatelet medication within the last month, recent cardiovascular events such as myocardial infarction, stroke or TIA, or signs for a silent central nervous system

(CNS) infarction on neuroimaging. For the replication sample, we only excluded patients with heparin treatment within the last month and patients with myeloproliferative disorders such as polycythemia vera or essential thrombocythemia. For TIA patients in the replication sample, a TIA was defined as acute neurologic symptoms lasting less than 24h, without evidence for brain ischemia in MRI or CT imaging. Blood of all ischemic stroke and TIA patients was drawn as soon as possible after hospital arrival and processed as described below. Beyond collection upon hospital arrival, blood samples were also collected on days 2, 3, 7 (or the day of discharge), and 90. Informed and written consent was obtained from all subjects included. The study was approved by the local ethics committee and conducted according to the Declaration of Helsinki.

3.2.2 Blood sampling and processing

Blood was drawn into a 9ml EDTA-tube using 21-gauge needles and incubated at room temperature for 30 minutes. The samples were centrifuged for 10 minutes at 2000*g*, followed by 15 minutes at 2500*g*. The samples were kept at -80°C.

3.2.3 Platelet spike-in experiment

This experiment was adapted from a protocol published by Kaudewitz et al [113]. Blood from 4 healthy volunteers was drawn into a 9ml EDTA-tube and incubated at room temperature for 30 minutes. To eliminate erythrocytes, the samples were 'centrifuged at 190*g* for 30 minutes. All centrifugations were performed at room temperature without brake' [113]. The supernatant was transferred into a different 5ml Falcon tube and centrifuged at 280*g* for 10 minutes at room temperature without brakes, in order to pellet white blood cells. The supernatant was again transferred into another Falcon tube, and the plasma was mixed by pipetting up and down several times to assure that remaining cells were spread equally throughout the plasma sample. The volume of this plasma sample (so-called platelet-rich plasma, PRP) was quantified, and 200µl of this PRP were transferred into a separate Falcon tube and stored on ice. The remaining PRP was pipetted back onto the cell pellet, to ensure that platelets that may already be pelleted remain in the sample. The sample was centrifuged at 1180*g* for 10 minutes under the same conditions as previously described, pelleting the platelets. 'The resulting supernatant was PPP' [113]. PPP was transferred into a different Falcon tube, and 200µl of PPP were stored on ice. The remaining pellet, which contained platelets and white blood cells, was dissolved in phosphate-buffered saline (PBS). The solution was transferred into a glass FACS tube and centrifuged at 1500*g* for 10 minutes at 15°C, now using slow brakes. The supernatant was discarded. In

order to ensure that the plasma sample contained no cells other than platelets a magnetic activated cell sorting (MACS) cell separation was performed as described below. 'The final platelet pellet was resuspended in 1/20 of the volume of original PRP to obtain a 20x stock platelet solution. The 20x platelet solution was then spiked back into the PPP from the same donor to achieve 200%, 100%, 50% and 5% spike-ins' [113]. miRNA expression levels in the different samples were analyzed via RNA-isolation, complementary DNA (cDNA) synthesis, and RT-qPCR as described above. In order to quantify platelet contribution to plasma expression levels of a miRNA, each threshold cycle value (C_t-value) of the spike-in samples (5%, 50%, 100%, 200%) was divided by the PRP value, and the 100% sample was compared to the PRP sample serving as an intrinsic control of the experiment.

5%	199.5µl PPP + 0.5µl of pellet
50%	195µl PPP + 5µl of pellet
100%	190µl PPP + 10µl of pellet
200%	180µl PPP + 20µl of pellet

Table 2: Spike-in of platelets in platelet-poor plasma
Spike-in of various amounts of platelets into PPP.

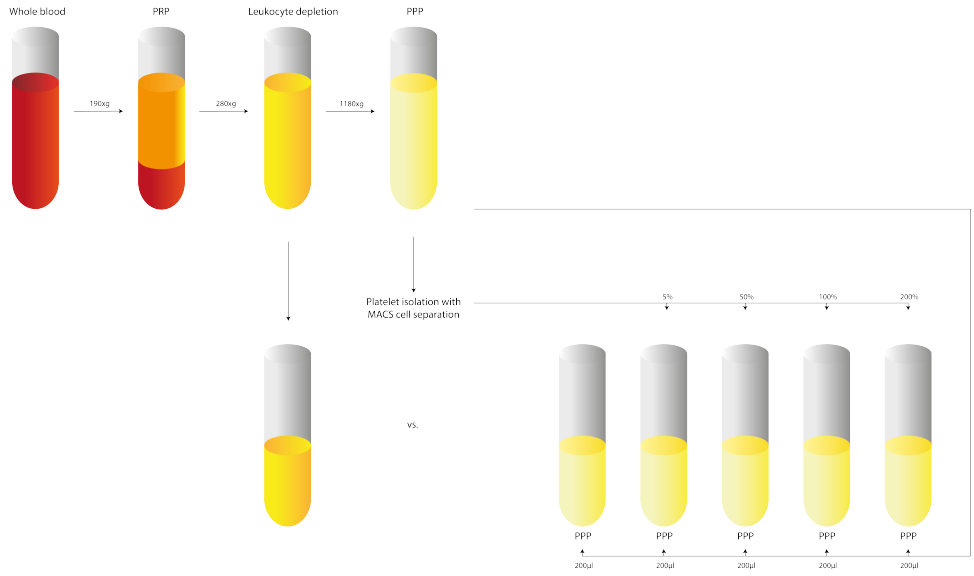

Figure 6: Platelet spike-in experiment
Analysis of platelet contribution to circulating miRNA plasma levels. MACS = magnetic activated cell sorting. PRP = platelet-rich plasma. PPP = platelet-poor plasma.

3.2.4 MACS cell separation

3ml of MACS running buffer were added to the cell pellet obtained in the platelet spike-in experiment. The sample was centrifuged at 1500*g* for 10 minutes at 15℃ with slow brakes. The cell pellet was resuspended in 80µl of MACS running buffer, and 10µl of Miltenyi Biotec CD61 MicroBeads for human samples were added. The solution was mixed by pipetting up and down several times, and then incubated for 30 minutes at 4℃, allowing the MicroBeads to attach to the CD61 receptor, which is ubiquitously expressed on platelets. After 30 minutes, the cells were suspended in 2ml of MACS running buffer and centrifuged at 1500*g* for 10 minutes at 15℃ once again, the supernatant was discarded. The MicroBead-labeled cells were dissolved in 300µl of MACS running buffer and added onto a 5ml tube rack. Two more glass tubes per sample were added for the positively and negatively selected cells. The automatic program 'Possel + Qrinse' was used, which performed a positive selection of magnetically labeled CD61-positive cells and a quick washing step between samples. The positively selected platelets were once more centrifuged at 1800*g* for 10 minutes at room temperature without brakes.

3.2.5 Animal experiments

Animal experiments were performed with the kind help of Uta Mamrak (AG Plesnila, ISD), Gemma Llovera Garcia and Vikramjeet Singh (AG Liesz, ISD). We used wild-type C57BL/6 mice from Charles River laboratories. Experimental stroke models were conducted as previously described [65]. In short, mice were randomly assigned to either the experimental stroke group or the control sham group. All mice were anesthetized using isoflurane with a mixture of 30% O_2 and 70% N_2O.

In the photothrombosis model, animals received 10µl/g body weight of 1% Rose Bengal in PBS intraperitoneally, and the brain was then locally illuminated by a fiber optic laser in order to induce a thrombotic stroke. In the distal middle cerebral artery occlusion (dMCAo) model, the middle cerebral artery (MCA) was permanently occluded by high-frequency electrocoagulation.

Blood sampling and processing were performed by a blinded experimenter. For final blood collection, mice were anesthetized using ketamine (120mg/kg) and xylazine (16mg/kg). Blood was collected by puncturing the right ventricle using 25-gauge needles. Blood was immediately drawn into EDTA-tubes. Samples were incubated for 30 minutes at room temperature, then centrifuged at

2000*g* for 10 minutes and for 2500*g* for 15 minutes at 15°C. Samples were kept at -80°C. A perfusion with 20ml of saline was performed with the mice used for fluorescent in-situ hybridization. The brain was extracted, frozen in an isopentane solution for 10 minutes and then stored at -80°C until further processing [114].

All experiments were approved by the governmental committee of upper Bavaria and conducted according to the guidelines for the use of experimental animals.

3.2.6 RNA isolation and cDNA synthesis

miRNA was isolated using the miRCURY RNA-Isolation Kit for Biofluids (Exiqon, Vedbaek, Denmark). RNA was extracted from 200µl per human plasma sample and 100µl per mouse sample. Following the manufacturer's recommendation, we added 60µl lysis buffer, 1.25µl glycogen to increase nucleic acid recovery [115] and Spike-ins (Exiqon Spike-ins UniSp2, 4 and 5) to each sample. 20µl of precipitation buffer provided in the kit was added. The last lysis step included a treatment with 270µl of isopropanol. Plasma was transferred onto provided filtered columns, followed by two washing steps with washing solutions I and II. Plasma samples were treated with 50µl DNAse provided in the kit and incubated for 15 minutes, followed by three identical washing steps. The remaining RNA on the column was diluted in 50µl of nuclease-free water, and either stored at -80°C or immediately processed for cDNA synthesis. Since most ischemic stroke patients received heparin during hospitalization, samples for longitudinal analysis were treated with heparinase for one hour before starting RNA isolation [116, 117].

cDNA synthesis was performed using the Exiqon Universal cDNA Synthesis Kit II. The samples acquired in the previous RNA-isolation were diluted with 100µl nuclease-free water. 3µl of each sample were transferred into Eppendorf DNA-LoBind tubes. Each experiment included a non-template control (water) and an RT- control, which lacked reverse transcriptase. To each sample, 1µl of Spike-in 6 (Exiqon Uni-Sp6 LNA PCR Primer set) was added as well as 2µl of reaction buffer (provided in the kit, including deoxynucleotide triphosphates (dNTPs), buffer, and cDNA-synthesis primers) and 3.5µl of nuclease free water. Samples were briefly centrifuged at 2000*g* for a few seconds to spin down and mix ingredients. Samples were then incubated at 42°C for 1 hour and subsequently incubated at 95°C for 5 minutes and stored at -80°C for long-term storage or at -20°C for short-term storage until proceeding to RT-qPCR analysis.

3.2.7 qPCR

cDNA samples were diluted in 150µl of nuclease-free water. 4µl of each sample, 5µl of ExiLENT SYBR Green Master Mix and 1µl of Primer (Exiqon microRNA LNA PCR Primer Set) were pipetted into a well on a 386-well qPCR plate. Samples were analyzed using a Roche Light Cycler.

Denaturation	95℃, 10 minutes, ramp rate 4.8 ℃/s
Amplification	45 cycles: 95 ℃, 10 seconds, ramp rate 1.6 ℃/s 60 ℃, 1 minute, ramp rate 2.5 ℃/s
Melting curve	Continuously up to 95℃, ramp rate 0.11 ℃/s
Cooling	37℃, 1 second, 2.5 ℃/s

Table 3: qPCR cycles and temperatures

C_t-values of spike-in UniSp4 were used to normalize the results, by using a median normalization method in the derivation sample, and a $\Delta\Delta C_t$ normalization in the replication sample. Samples with C_t-values greater than 35 were set to 40. When analyzing samples of the same cohort on more than one qPCR plate, recombinant miRNA with determined copy numbers were used to adjust the results.

3.2.8 Analysis of differences in blood sampling procedure

To identify ideal experimental conditions, we assessed the impact of various factors on miRNA yield. A 9ml blood sample of a healthy control person was drawn into an EDTA-tube and separated into 12 separate 200µl samples. Subsequently, each of them was treated differently. Samples were processed differently regarding incubation temperature, centrifugation steps to deplete platelets and an additional centrifugation step prior to starting RNA isolation according to Figure 7. RNA-Isolation, cDNA synthesis and RT-qPCR were performed as described above, and expression levels for miR-126-3p, miR-126-5p, miR-223-3p (high levels in platelets, see results) and miR-122-5p (not present in platelets, see results) were compared. Since all initial samples derived from the same plasma probe, miRNA levels should be the same in all samples, and differences would indicate confounding factors in our protocol.

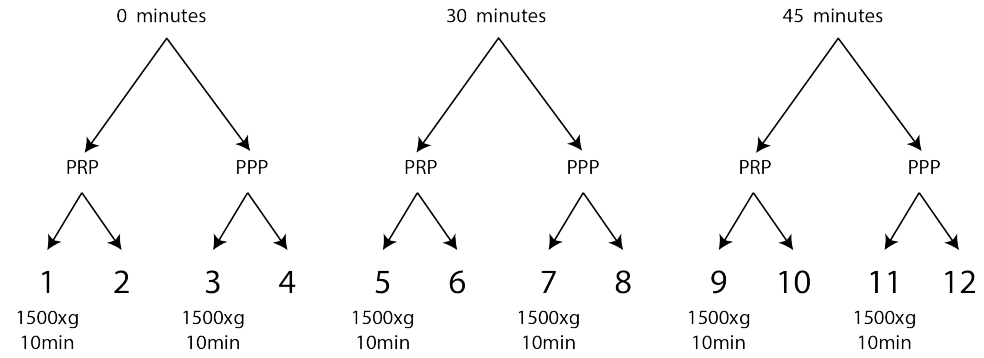

Figure 7: Different treatment of 12 samples with different protocols
The twelve samples differ in three factors: time of incubation immediately after blood draw, platelet presence in the sample, and an extra centrifugation step on day 2 before starting RNA-isolation. PRP = platelet-rich plasma. PPP = platelet-poor plasma.

3.2.9 Fluorescent in-situ hybridization

Fluorescent in-situ hybridization (FISH) was performed using a three-day protocol, based on a previously published protocol by Chaudhuri et al [118]. For the FISH analysis, we combined cell staining of neurons and astrocytes with hybridization of miR-126-3p and miR-126-5p. Brain samples were cut into 5µm sections using a Microtome. Four sections were fixed onto one slide and stored at room temperature in the dark.

The slides were deparaffinized according to the following scheme:

- 'Xylene, 3 times, 5 min each
- 100% ethanol, twice, 5 min each
- 70% ethanol (diluted in DEPC-treated water), once, 5min
- 50% ethanol (diluted in DEPC-treated water), once, 5 min
- DEPC water, twice, 3 min each' [118].

Antigen retrieval was performed by incubating the samples 'in 0,01M citrate buffer pH = 6,4, for 40 min at 90℃' [118] followed by 20 minutes at room temperature. Before proceeding to ethylcarbodiimide (EDC) treatment, samples were washed 'with TBS [(Tris-buffered saline)], 3 times, 3 min each' [118]. Samples were incubated twice for 10 minutes each in Methylimidazole buffer at room temperature, followed by a one-hour treatment with EDC solution in a TBS-humidified chamber. Samples were washed with TBS/0.2% Glycine solution for 5 minutes, followed by two washing steps in pure TBS for 5 minutes each.

In the prehybridization step, the hybridization chamber was prepared 'by placing 1X SSC-soaked Kimwipes at the bottom of the chamber' [118]. The hybridization buffer was heated up to 37℃ and carefully pipetted onto the slides. The slides were incubated for one hour at room temperature in an SSC-humidified chamber.

The hybridization mix was prepared: Exiqon LNA FISH probes for the miRNAs of interest, 5'- and 3'-digoxigenin labeled, were diluted in hybridization buffer up to a total concentration of 4pmol in 2µl. The mix was incubated for 5 minutes at '65℃ for 5 min to ensure denaturation of probes' [118] and then pipetted onto the slides. The slides were incubated in an SSC-humidified chamber overnight at specific hybridization temperatures (which is approximately 30℃ lower than the specific DNA melting temperature):

	Specific DNA melting temperature	**Incubation temperature**
126-5p	81℃	51℃
126-3p	84℃	54℃
Scramble probe	87℃	57℃

Table 4: Incubation temperatures for miRNA FISH probes

The scramble probe, which only contained fragments, was used as an intrinsic negative control. The slides were covered with wax and stored overnight.

The next morning, the slides were washed according to the following:

- 'Three times for 20 min each with 2x SSC at 42°
- Twice for 20 min each with 0.2x SSC at 42°' [118]

The samples were washed in a solution of TBS/0.1% Tween for 15 minutes.

For blocking, the samples were incubated in blocking buffer for one hour at room temperature in a PBS-humidified chamber. The primary antibody was diluted using blocking buffer at specific concentrations:

Antibody	Dilution
α-NeuN mouse (to stain neurons)	1:100
α-GFAP mouse (to stain astrocytes)	1:500
α-digoxigenin POD (to bind 3'- and 5'-DIG labeled sites)	1:100

Table 5: Dilution of antibodies

The slides were left at 4℃ overnight, allowing the antibodies to attach their specific sites.

On the third day, the slides were washed 'twice in TBS for 2 min each at room temperature' [118]. The secondary antibody, anti-mouse Alexa Flour 488 (green, binding primary antibody for cell staining) was diluted in TBS at 1:500. The slides were incubated for 1 hour in a TBS-humidified chamber and washed twice for 2 minutes in TBS again. The Cy3-TSA secondary antibody (against α-digoxigenin POD) was applied at a concentration of 1:100. The slides were incubated again for 10 minutes in a TBS-humidified chamber and washed three times for 5 minutes each in TBS. Subsequently, the slides were treated with 4',6-Diamidino-2-Phenylindole (DAPI) at a dilution of 1:5000. The slides were washed for 5 minutes in TBS, then quickly rinsed with DEPC-H_2O and covered using 22x50mm cover glasses and Flouromount mounting cover. The samples were stored at 4℃ in the dark until the Confocal microscope analysis.

Confocal microscopy was performed and analyzed using the ZEN Software (Zeiss). Images were captured and extracted from Z-stacks. Maximum intensity projection function was applied.

3.2.10 Statistics

Data were displayed as median±interquartile range or mean±standard error of the mean. Differences in demographic factors and vascular risk factors in the derivation and replication sample were analyzed using Fisher's exact test, student's *t*-test, and Chi^2-test and ANOVA, respectively. The Mann-Whitney-U-test was used for comparison of two groups, while more than two groups were compared using 1-way ANOVA or Kruskal-Wallis-Test followed by Dunn's multiple comparison test. Matched data in the platelet spike-in experiment were analyzed using the Friedman-Test. For multivariate analysis, Bonferroni correction for multiple testing was used if indicated. Covariates in derivation and replication sample were identified by backward stepwise regression and all results were adjusted

with a linear multivariate model. In all experiments, a p-value < 0.05 was used to determine statistical significance. Receiver operating characteristics were created using random forest classification. All statistical analyses were performed by using GraphPad Prism Version 6.0 for Windows and 'R', version 3.4.0.

4 Results

4.1 Analysis of platelet-associated circulating miRNAs in ischemic stroke patients

4.1.1 miRNA selection based on literature

Recent studies suggested that expression levels of various miRNAs might correlate with platelet function and activation. Since platelet aggregation and activation play an essential role in ischemic stroke pathophysiology, we decided to focus on platelet-associated miRNAs in order to investigate miRNA expression patterns and changes in plasma levels of circulating miRNAs after ischemic stroke. Thus, we performed a literature search with search terms including 'microRNA' or 'miRNA' and 'platelet' or 'thrombocyte'. Finally, we selected 12 miRNAs that had been described to correlate with platelet function and activation in literature: miR-21-5p, miR-24-3p, miR-30b-5p, miR-92a-3p and miR-191-5p based on a publication of Edelstein et al. in 2013 [119], miR-20b-5p [113], miR-150-5p [120], miR-155-5p [121] and miR-197-3p [122], as well as miR-126-3p and miR-126-5p [106, 107], and miR-223-3p. miR-223-3p has been shown to bind the 3'-UTR of human $P2Y_{12}$-receptor mRNA, thus regulating receptor levels and platelet function [123], and shows significantly decreased plasma levels in humans treated with clopidogrel, an ADP-antagonistic drug [124]. In our platelet-related experiments, we used miR-223-3p as a positive control for platelet association when analyzing other miRNAs. We also used miR-122-5p as a negative control, since this miRNA has been suggested to be liver-specific and not to be expressed in any other tissues [125].

In order to identify miRNAs that are expressed in a range of plasma levels that could be reliably detected with our laboratory devices and experimental protocols, we first quantified levels of the 12 miRNAs in a blood sample of a healthy human volunteer. Since accuracy of qPCR measurement drastically decreases above an average C_t-value of 35 [126], we only included the eight miRNAs that were detected with a C_t-value below 35.

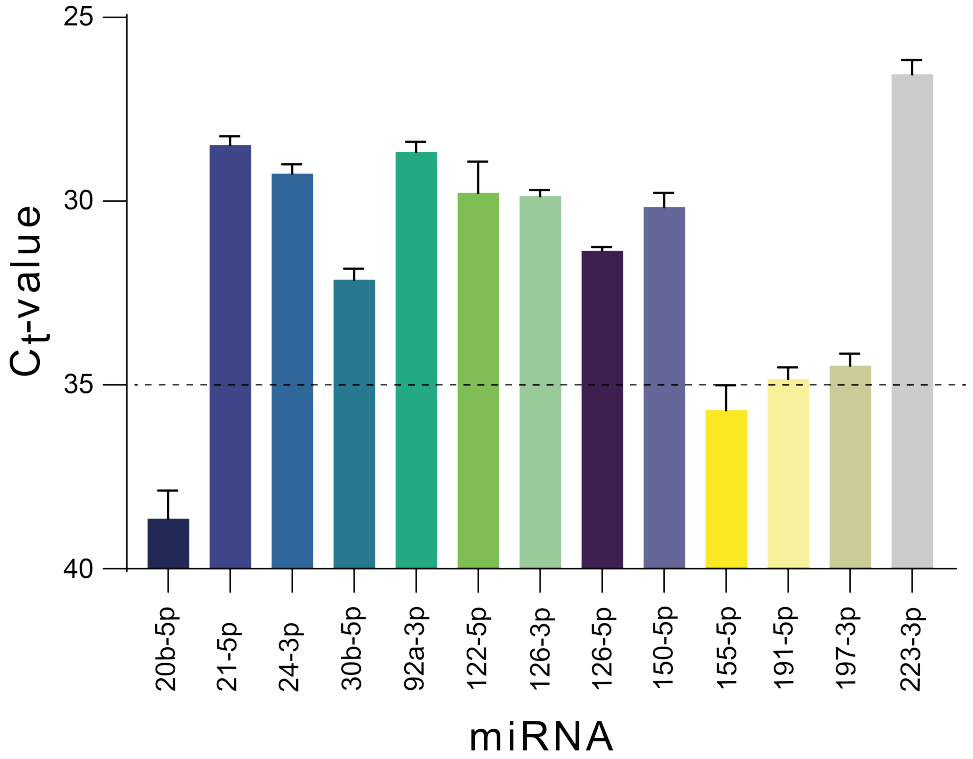

Figure 8A: miRNA plasma levels in healthy human volunteers
Plasma levels of the 12 miRNAs selected in literature review in healthy human volunteers. Mean±SEM, results from five plasma samples of a single healthy human volunteer.

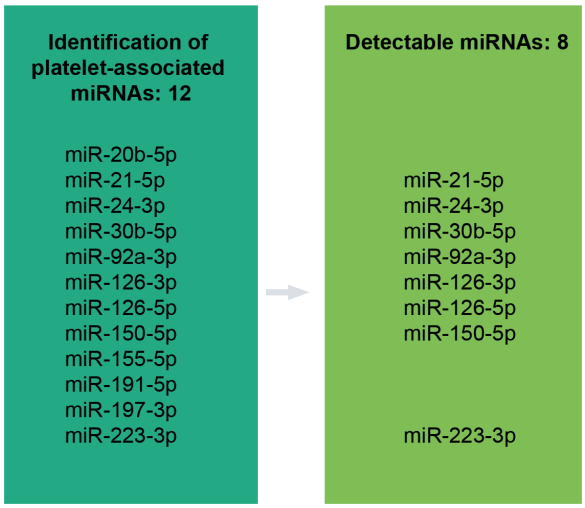

Figure 8B: Selection of miRNAs
Selection of twelve miRNAs based on systematic review of literature. Eight of them were reliably detectable in human control plasma by RT-qPCR.

4.1.2 Platelets contribute to miRNA plasma levels

Next, we investigated to what extent platelets contribute to plasma levels of these miRNAs and thus performed a platelet spike-in experiment. In short, we isolated

platelets from blood samples of healthy volunteers, spiked back various numbers of platelets into PPP and analyzed miRNA levels in those plasma samples. Plasma levels of seven miRNAs increased significantly with increasing number of platelets added to platelet-poor plasma: miR-21-5p, miR-24-3p, miR-30b-5p, miR-92a-3p, miR-126-3p and miR-126-5p, as well as miR-223-3p. Liver-specific miR-122-5p did not show significant changes with the number of platelets spiked in. In addition, miR-150-5p levels did not show a significant increase with platelet numbers and was therefore excluded from further experiments.

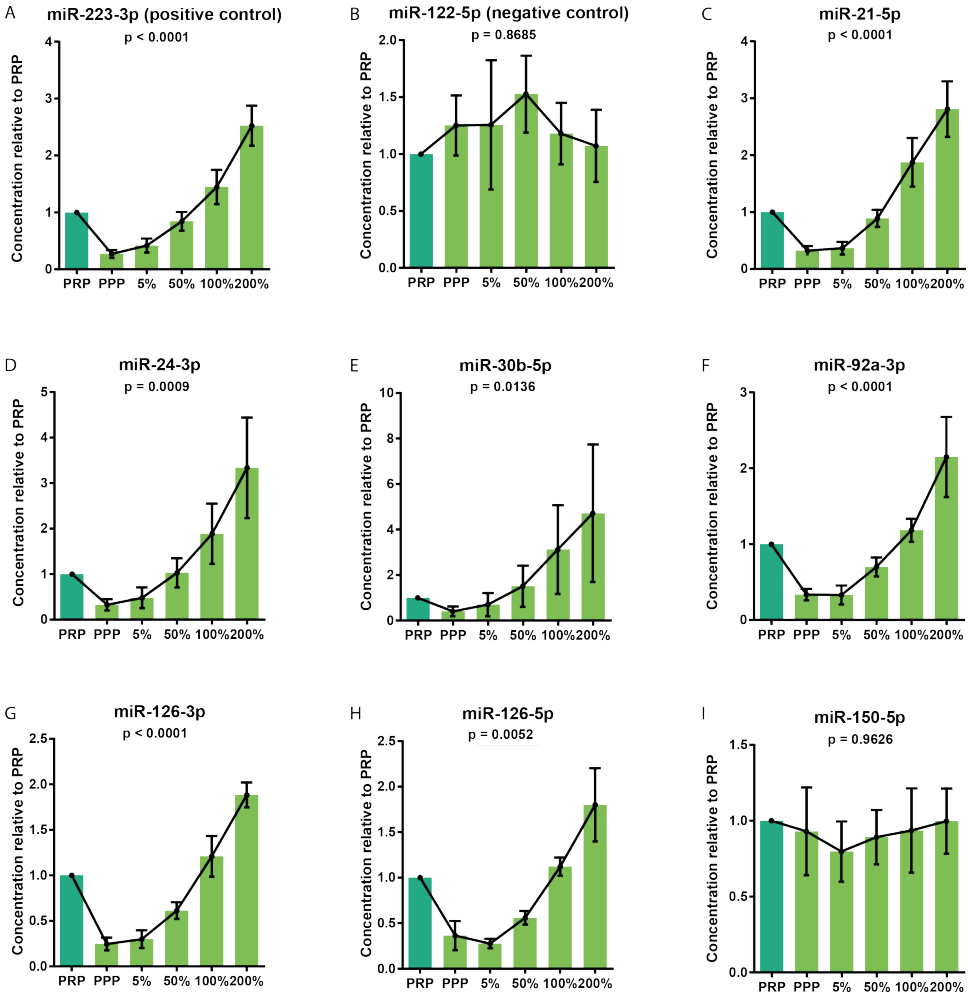

Figure 9A: miRNA levels increase with increasing platelet number

Positive control miR-223-3p shows a significant increase of circulating miRNA levels with the number of platelets spiked into platelet-poor plasma (A), while negative control miR-122-5p does not (B). miR-21-5p (C), miR-24-3p (D), miR-30b-5p (E), miR-92a-3p (F), miR-126-3p (G) and miR-126-5p (H) show a significant increase as well. miR-150-5p does not show a significant change in this experiment. X-axis: percentages of original platelet number spiked in. Friedman-Test. Mean±SEM, n=4. PRP = platelet-rich plasma, PPP = platelet-poor plasma.

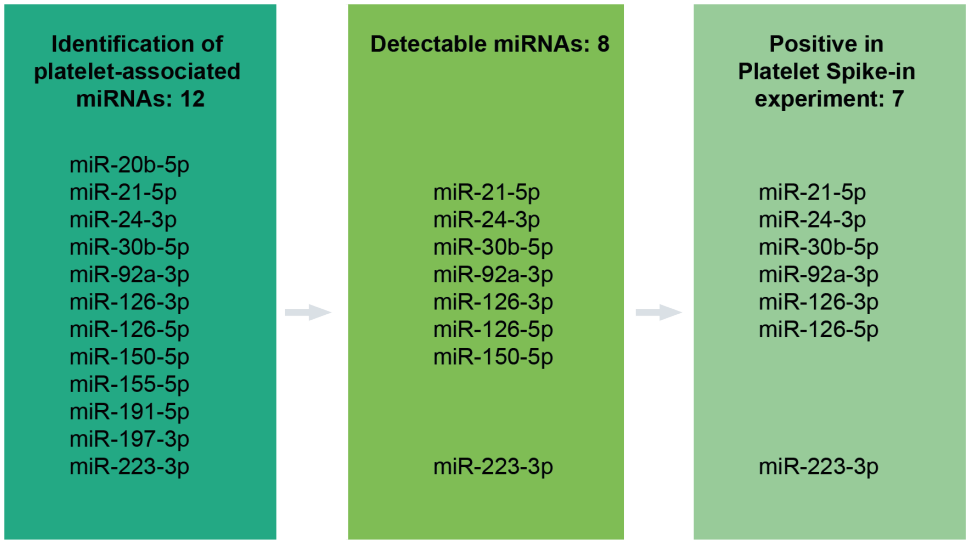

Figure 9B: Selection of miRNAs
Seven miRNAs showed a significant increase with platelet numbers spiked in in the platelet spike-in experiment.

4.1.3 Derivation sample

One of the main goals of our experiments was to test the hypothesis that platelet-associated miRNAs show higher levels in patients with ischemic stroke compared to healthy controls. After identifying seven miRNAs whose plasma levels increase with platelet numbers in the platelet spike-in experiment, we aimed to analyze whether those miRNAs would show changes in plasma levels of ischemic stroke patients. The derivation sample included 60 stroke patients as well as 60 healthy control subjects. The mean time from symptom onset to hospital arrival in the ischemic stroke patient group was 5.4±4.3 hours and the mean infarct volume was 18.6±40.8 ml (Table 6).

Characteristics	**Healthy Controls**	**Ischemic Stroke**	***p*-value**
Demographics			
Total	60	60	
Age [years], mean (*SD*)	70.7 (9.3)	74.7 (12.5)	0.050
Female	34 (56.7)	26 (43.3)	0.201
Vascular Risk factors			
Hypertension	38 (63.3)	50 (83.3)	**0.022**
Smoking History	21 (35)	28 (46.7)	0.265
Hypercholesterolemia	17 (28.3)	18 (30)	1
Obesity	7 (11.7)	11 (18.3)	0.444
Family History	4 (6.7)	11 (18.3)	0.095
Antiplatelet therapy	16 (26.7)	18 (30)	0.840
Previous TIA/stroke/MI	2 (3.3)	13 (21.7)	**0.004**
Ischemic Stroke	n/a	60 (100)	n/a
T_{Mean} [hours] Symptom onset –> ED (*SD*)	n/a	5.4 (4.3)	n/a

Table 6: Derivation sample
Demographic characteristics and vascular risk factors of stroke patients and healthy control subjects included in the derivation sample. Fisher's exact test, except for Age (*t*-test). All values presented in n (%) unless indicated otherwise. *SD* = standard deviation, MI = myocardial infarction, ED = arrival at emergency department.

Plasma levels of five miRNAs were significantly elevated in acute ischemic stroke patients compared to healthy controls in the derivation sample: miR-21-5p (p=0.003), miR-24-3p ($p < 0.0001$), miR-126-3p (p=0.0004), miR-126-5p (p=0.0001) and miR-223-3p (p=0.0001). miR-122-5p, the negative control, did not show any alterations, nor did miR-30b-5p and miR-92a-3p.

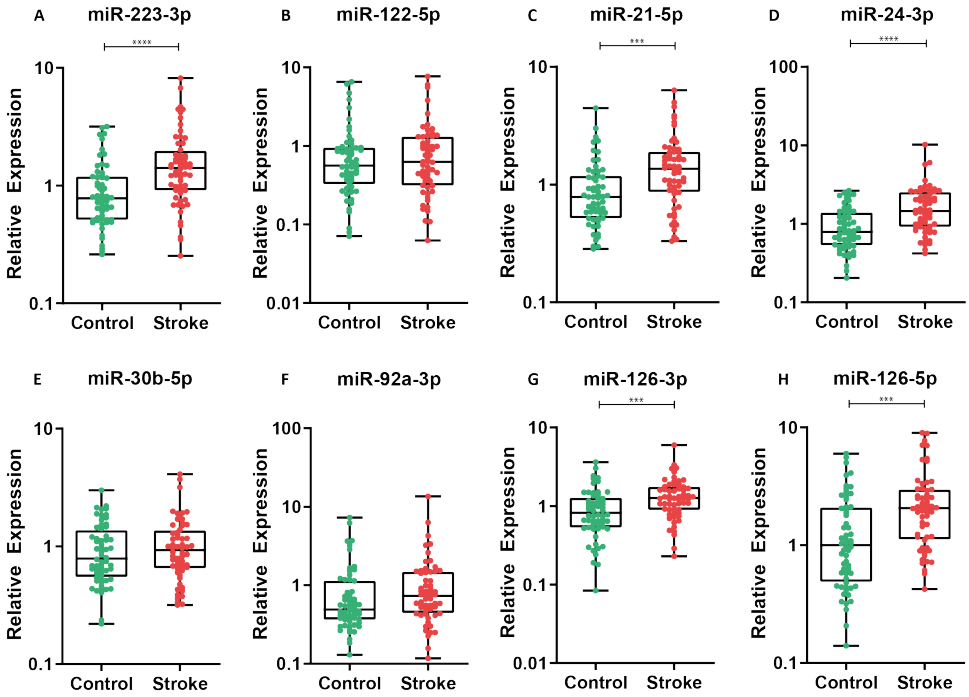

Figure 10: Derivation sample
Plasma levels of miR-223-3p (A, positive control), miR-21-5p (C), miR-24-3p (D), miR-126-3p (G) and miR-126-5p (H) were significantly elevated in acute ischemic stroke patients compared to healthy controls. miR-122-5p (B), which served as a negative control, was not altered. Mann-Whitney-U-Test, median±interquartile range, bars display 10th and 90th percentile, n=60 per group.

Both hypertension and previous cardiovascular or cerebrovascular events were significantly more prevalent in the ischemic stroke group compared to the control group and were identified as potential confounders using backward stepwise regression analysis. However, adjusting the results using a linear multivariate model did not result in major changes of the *p*-values indicated above.

4.1.4 Replication of elevated plasma miRNA levels after ischemic stroke and TIA

In order to validate the results in an independent and larger patient collective, 200 acute ischemic stroke patients, 100 healthy controls and 71 patients with TIA were recruited for the replication sample. In this group, the mean time from symptom onset to hospital arrival was 5.0±4.6 hours for stroke patients and 6.0±6.4 hours for TIA patients, respectively.

Characteristics	**Healthy Controls**	**TIA**	**Ischemic Stroke**	***p*-value**
Demographics				
Total	100	71	200	
Age [years], mean (*SD*)	65.6 (13.4)	74.6 (12.4)	74.1 (13.4)	**< 0.0001**
Female	65 (65)	38 (53.5)	87 (43.5)	**0.003**
Vascular Risk factors				
Hypertension	35 (35)	54 (76.1)	157 (78.9)	**< 0.0001**
Smoking History	35 (35)	30 (45.5)	74 (40.4)	0.256
Hypercholesterolemia	21 (21)	34 (47.9)	55 (27.8)	**0.004**
Obesity	19 (19.2)	13 (21.7)	28 (20.9)	0.927
Family History	9 (9)	9 (13.4)	23 (12.8)	0.006
Antiplatelet therapy	18 (18)	33 (46.5)	66 (33.5)	**0.0004**
Previous TIA/stroke/MI	7 (7)	35 (49.3)	65 (32.8)	**< 0.0001**
Ischemic Stroke	n/a	n/a	200 (100)	n/a
T_{Mean} [hours] Symptom onset –> ED (*SD*)	n/a	6.0 (6.4)	5.0 (4.6)	0.176

Table 7: Replication sample
Demographic characteristics and vascular risk factors of stroke patients, TIA patients and healthy control subjects included in the trial. Chi^2-Test, except for Age (ANOVA) and T_{Mean} symptom onset –> ED (*t*-test). All values presented in n (%) unless indicated otherwise. *SD* = standard deviation, MI = myocardial infarction, ED = arrival at emergency department.

Applying multivariable linear regression analysis adjusting for potential confounders age, sex, hypertension, antiplatelet therapy and previous cardiovascular or cerebrovascular events, all five miRNAs that had shown significantly elevated plasma levels in ischemic stroke patients in the derivation sample, showed elevated plasma levels in the replication sample as well: miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p, and miR-223-3p (all: $p < 0.0001$). Furthermore, plasma levels of miR-126-3p and miR-223-3p were significantly elevated in ischemic stroke patients compared to TIA patients, while the remaining miRNAs did not show significant differences between those two groups.

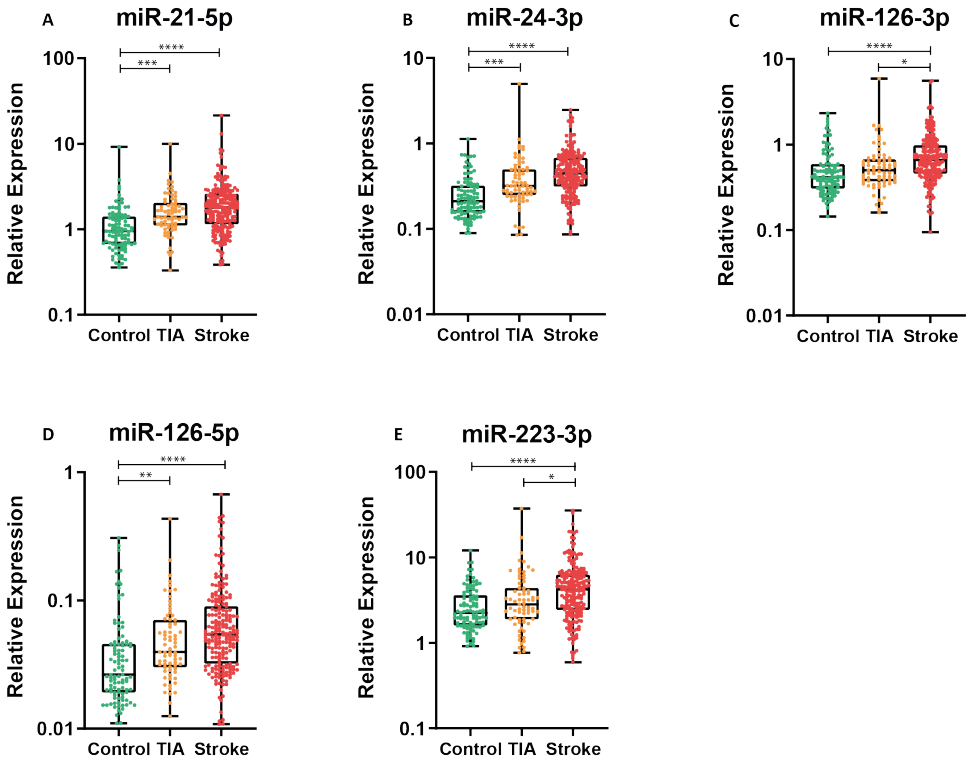

Figure 11A: Replication sample

All five miRNAs showed significantly elevated plasma levels in the stroke group compared to the healthy control group (all: $p < 0.0001$). miR-126-3p (C) and miR-223-3p (E) levels were also significantly elevated in acute ischemic stroke patients compared to TIA patients. Mann-Whitney-U Test, median±interquartile range, bars display 10th and 90th percentile. Controls: n=100, TIA: n=71, Stroke: n=200. TIA = transient ischemic attack.

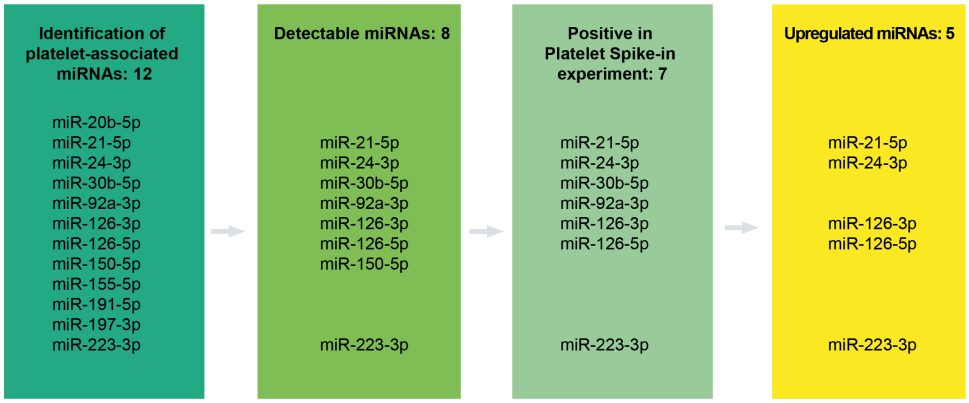

Figure 11B: Selection of miRNAs

Five platelet-associated miRNAs showed significantly elevated plasma levels in acute ischemic stroke patients compared to healthy control subjects.

4.1.5 miRNA plasma levels return to baseline levels quickly following ischemic stroke

After we detected a significant increase in plasma levels of several platelet-associated circulating miRNAs on the day of symptom onset, we aimed at further investigating the time course of plasma levels of those miRNAs, in order to assess whether those changes are linked to the acute stage of stroke only, or if plasma levels are further affected by secondary processes such as inflammation or tissue reorganization. Thus, we performed longitudinal analyses of miRNA levels during the acute phase (days 2, 3 and 7 or discharge day), and at 90 days after stroke.

	Day 1 ED	**Day 2** Stroke Unit	**Day 3** Stroke Unit	**Day 7 (D/C)** Stroke Unit	**Day 90** ISD Outpatient Clinic
Patients	40	40	40	38	17
Controls	20				

Table 8: Longitudinal follow-up of acute ischemic stroke patients
Number of patients included in the longitudinal analysis, time course and sites of recruitment. ED = emergency department. ISD = Institute for Stroke and Dementia Research. D/C = discharge.

For all five miRNAs except for miR-24-3p, we measured significantly elevated plasma levels on day 1 that rapidly returned to control levels on day 2 and showed no significant changes in the samples analyzed up to day 90. miR-24-3p remained significantly elevated up to day 3 after stroke, and then returned to baseline levels.

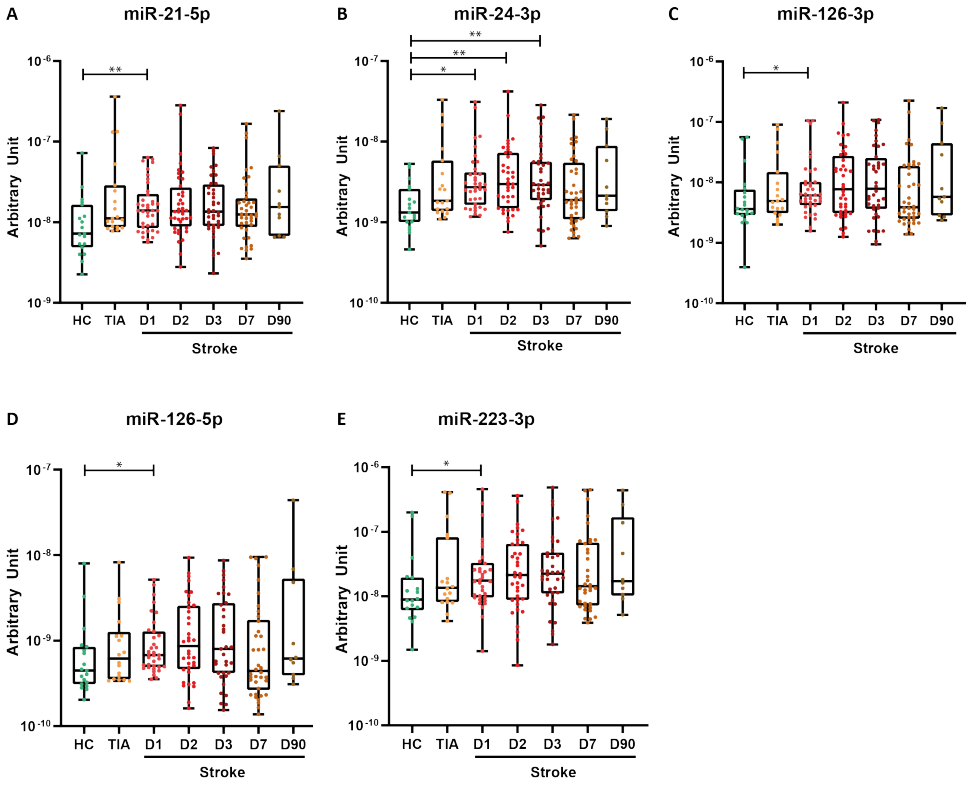

Figure 12: Longitudinal analysis of miRNA plasma levels after stroke
All five miRNAs showed significantly elevated plasma levels on day 1 after stroke. miRNA levels returned to normal on day 2, except for miR-24-3p (B) levels, which only returned to baseline after day 3. 1-Way-ANOVA for multiple comparisons, median±interquartile range, bars display 10th and 90th percentile. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Controls: n=20, TIA: n=20, D1, D2, D3: n=40, D7: n=38, D90: n=17. HC = healthy controls, D1-D90: day 1 – day 90.

4.1.6 Prior acetylsalicylic acid treatment has impact on miRNA plasma levels

Several of the patients and control subjects included in our study had previously been treated with acetylsalicylic acid (ASA). Here, we aimed at investigating the impact of prior antiplatelet treatment on plasma levels of the platelet-linked miRNAs. We selected a group of stroke patients and healthy controls from the derivation sample that had been treated with ASA in the past seven days and compared miRNA levels in both groups, as well as plasma levels in an equally sized group of stroke patients and healthy controls without prior ASA treatment. The ASA group included 19 patients and 15 healthy controls, the non-ASA group 21 patients and 25 healthy controls. When comparing ischemic stroke patients and healthy subjects without prior ASA treatment, all five miRNAs showed significantly elevated plasma levels in the stroke group. When comparing ischemic stroke patients and healthy controls with prior ASA treatment, miR-21-5p and miR-223-3p did not.

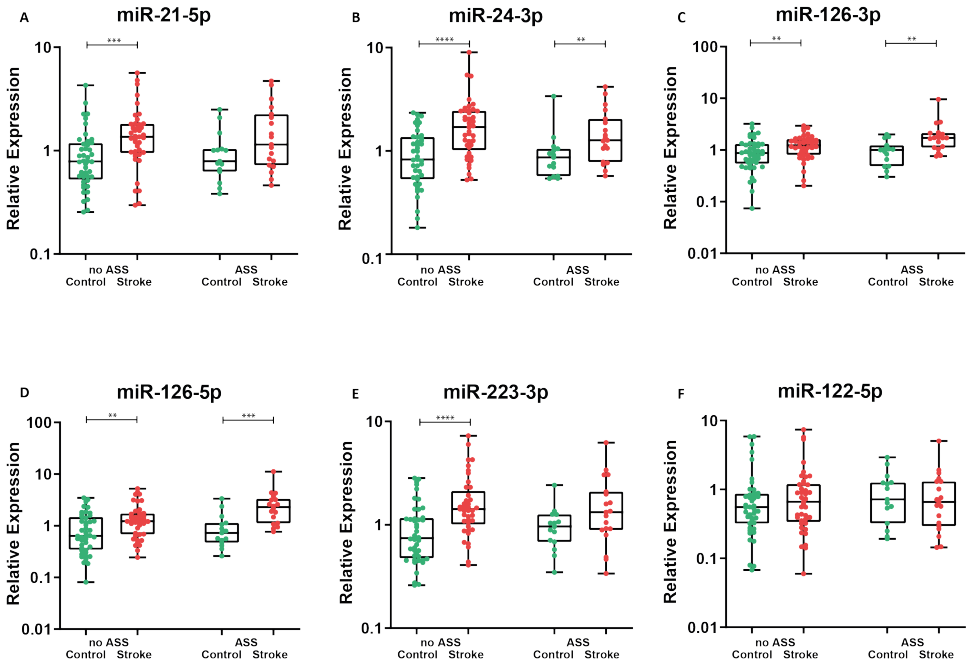

Figure 13: miRNA plasma levels in patients treated with and without ASA
miRNA plasma levels in ischemic stroke and healthy controls divided into two groups, one of which had been taking ASA prior to the stroke. miR-21-5p (A) and miR-223-3p (E) showed significantly elevated plasma levels in the group without ASA, but not in the ASA group. miR-24-3p (B), miR-126-3p (C) and miR-126-5p levels (D) were significantly increased in both no-ASA and ASA group. Mann-Whitney-U Test, median±interquartile range. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. No ASA: Controls: n=25, Stroke: n=21. ASA: Controls: n=15, Stroke: n=19. ASA = acetylsalicylic acid.

4.1.7 Diagnostic utility of miRNAs

To evaluate the utility of the identified miRNAs as diagnostic biomarkers in the acute setting we performed a receiver operating characteristic (ROC) analysis. The combination of miR-21-5p, miR-24-5p, miR-126-3p, miR-126-5p, and miR-223-3p differentiated between ischemic stroke patients and healthy controls with an area under the curve (AUC) of 0.82, which corresponds to a sensitivity of 84% and a specificity of 68%. The AUC for distinguishing ischemic stroke and TIA patients was 0.57 (sensitivity 39%, specificity 82%).

In a previous study [65], our group identified miR-125a-3p, miR-125b-3p and miR-143-3p as potential diagnostic biomarkers for acute ischemic stroke. The combined set of eight miRNAs, differentiating ischemic stroke patients and healthy controls, resulted in an AUC of to 0.94, and did improve specificity (92%) but not sensitivity (82%).

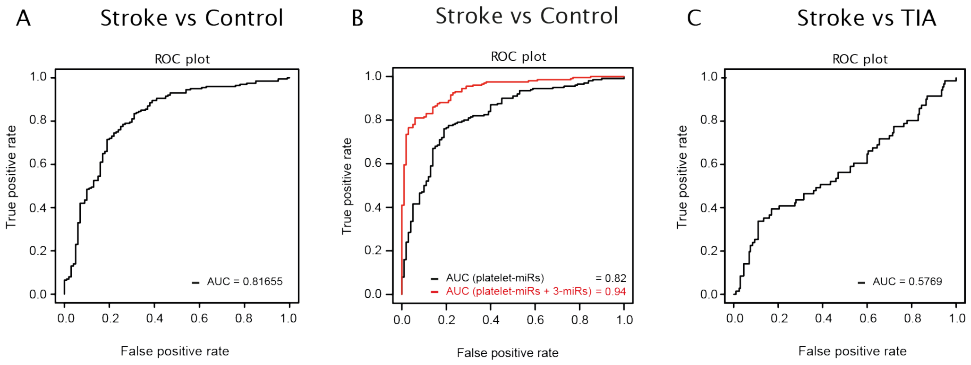

Figure 14: Receiver operating characteristics
Receiver operating characteristics for (A) ischemic stroke vs. healthy controls including miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p, and miR-223-3p, (B) ischemic stroke vs. healthy controls combining the five miRNAs with miR-125a-3p, miR-125b-5p and miR-143-3p that were identified in a previous study done by our group. (C) Ischemic stroke vs. TIA including miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p, and miR-223-3p. ROC = receiver operating characteristics, AUC = area under the curve. TIA = transient ischemic attack. miR = microRNA.

4.1.8 Influence of differences in blood sampling procedure

An important characteristic of a useful diagnostic biomarker is being reliable regardless of experimental conditions and laboratory setting [127]. In order to examine the impact of different working conditions such as incubation time, number of centrifugation steps, and platelet number present in the sample on the results of our experiments, we compared 12 different plasma samples of one single healthy human volunteer and treated each of them individually.

To significantly reduce the number of platelets in plasma samples, our RNA isolation protocol included an additional centrifugation step of 2500*g* for 15 minutes. We first compared plasma levels of miR-126-3p, miR-126-5p, as well as miR-223-3p and miR-122-5p with and without this step. miR-126-3p, miR-126-5p and miR-223-3p showed significantly higher plasma levels in the sample containing platelets, which was in line with the results of the platelet spike-in experiment.

The plasma samples of study patients in our experiments were stored at -80℃ until further usage. Our protocol included an extra centrifugation step at 1500*g* for 10 minutes immediately after thawing. Comparing expression levels of those samples with and without the centrifugation step did not show significant differences in plasma levels.

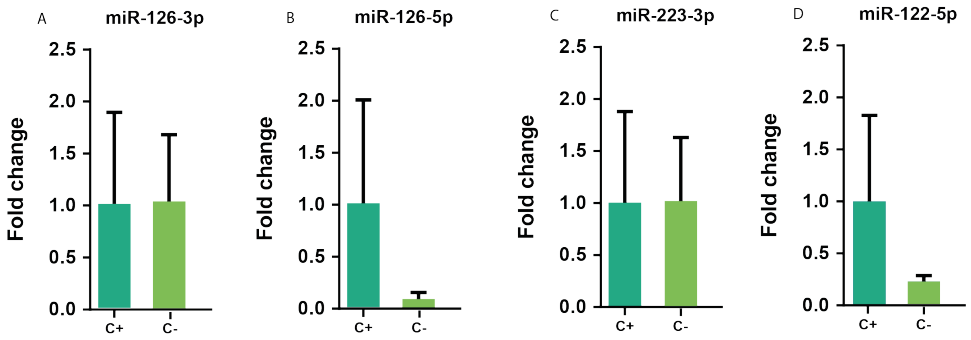

Figure 15A: Comparison of the extra centrifugation step vs. no centrifugation
Centrifugation step: 1500*g* for 10 minutes prior to RNA-isolation. miR-126-3p (A), miR-126-5p (B), miR-223-3p (C) and miR-122-5p (D) plasma levels in centrifuged plasma (C+) compared to plasma without extra centrifugation step (C-). None of the miRNAs showed significant changes with an extra centrifugation. Mann-Whitney-U-Test, Mean±SEM, n=6.

Next, we aimed at investigating the impact of differences in working speed on experimental results. The protocol for blood draw and sampling for study subjects included a 30-minute incubation step at room temperature. We divided the sample in three parts and left the samples at room temperature for different time periods (0 minutes, 30 minutes, and 45 minutes). There were no significant differences between the three time points in either of the four miRNAs.

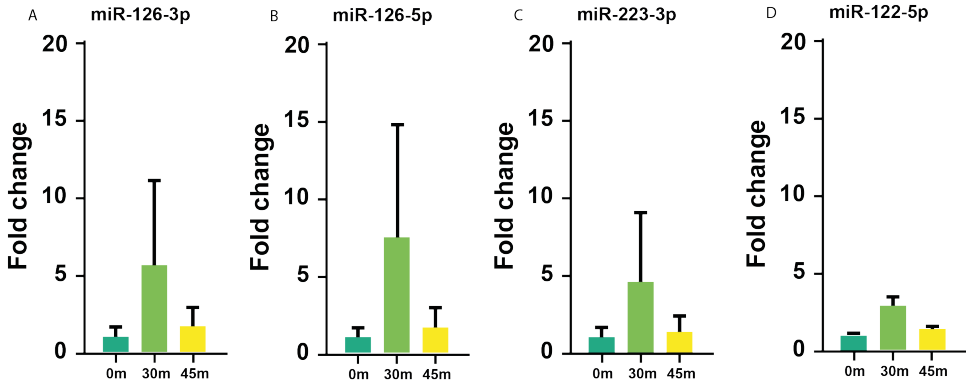

Figure 15B: Different incubating times for plasma samples before processing
miR-126-3p (A), miR-126-5p (B), miR-223-3p (C) and miR-122-5p (D) plasma levels after leaving plasma samples at room temperature for 0, 30 or 45 minutes prior to starting RNA isolation. No significant differences could be observed, but it was remarkable that RNA yield was highest after 30 minutes for all miRNAs. Kruskal-Wallis-Test, Mean±SEM, n=4.

4.2 Circulating miRNAs in experimental murine stroke models

4.2.1 miRNA plasma levels after induced murine stroke - photothrombosis

We considered the photothrombosis model as the most accurate experimental murine stroke model available for studying platelet function and aggregation in setting of an ischemic stroke, since it involves formation of a local thrombosis induced by a fiber optic laser. Here, we compared plasma levels of the seven miRNAs we initially selected for the platelet spike-in experiment, as well as negative control miR-122-5p, after photothrombotic stroke compared to a sham group.

None of the five miRNAs that were significantly altered in human stroke patients showed significant consistent alterations in murine plasma levels after stroke. miR-21-5p levels, which had been significantly elevated in ischemic stroke patients, even showed a significant decrease after photothrombosis-induced stroke in mice.

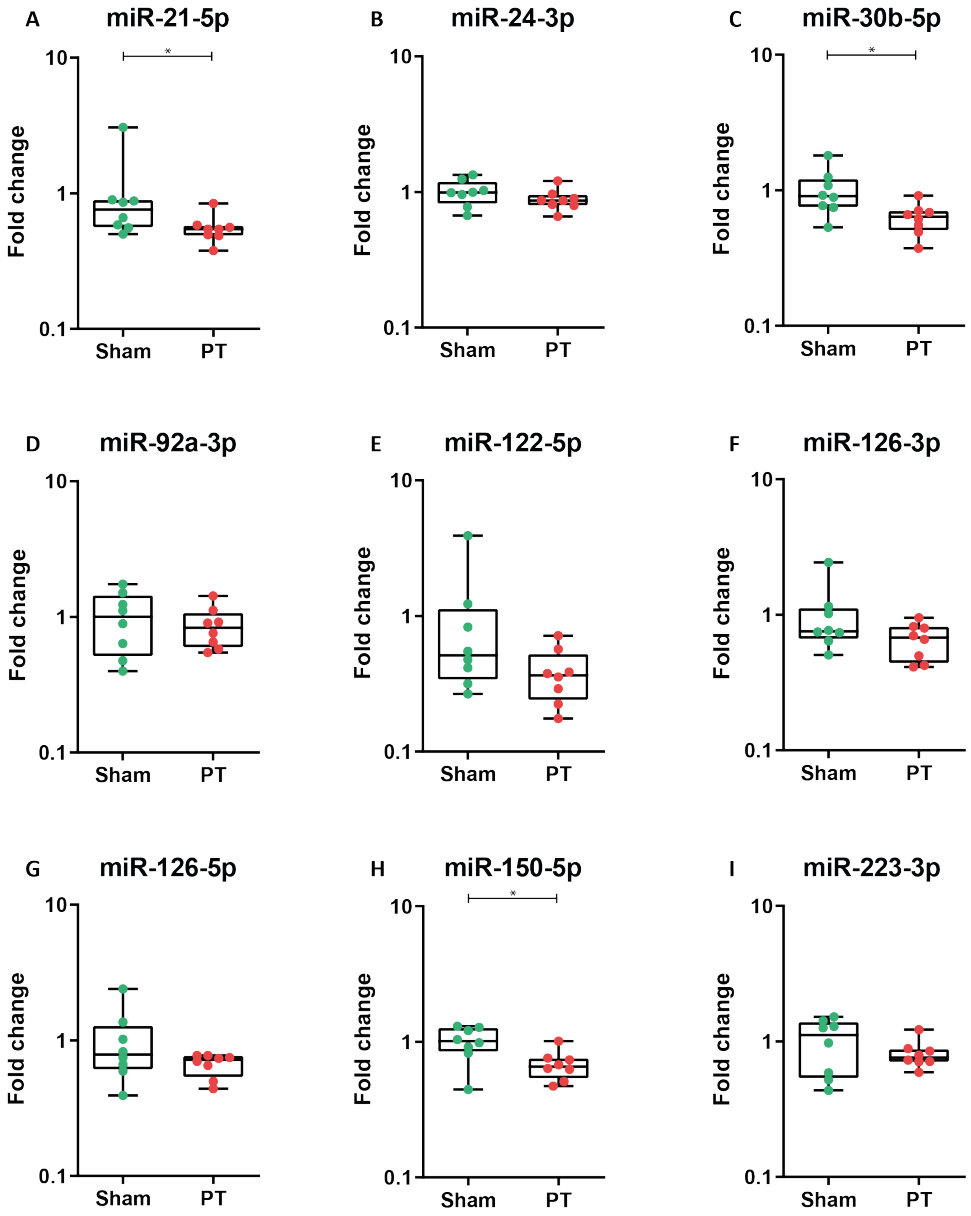

Figure 16: Photothrombosis model
Plasma levels of miRNAs in the photothrombosis stroke model, compared to a sham group. miR-21-5p (A, p=0.0207), miR-30b-5p (C, p=0.0148) and miR-150-5p (H, p=0.0281) showed a significant decrease in plasma levels 6 hours after murine stroke. Mann-Whitney-U-Test, Mean±SEM, n=8. PT = photothrombosis.

4.2.2 miRNA analysis in murine brain samples

To test the hypothesis that the identified miRNAs are indeed platelet-associated and not related to neurons or glial cell expression during an ischemic stroke, we aimed at investigating miRNA expression in the brain. In a first approach, we combined FISH with immunofluorescent labeling on brain sections of dMCAo-treated mice. We labeled neurons with NeuN, astrocytes with GFAP and miRNAs with probes in the same sample, to assess whether a cell-type specific

expression of miRNAs can be detected.

In all stainings, scramble miRNA 3'-5'-digoxigenin-labeled probes served as a negative control. Areas with enhanced miR-126-3p staining did not colocalize with NeuN-labeled neurons in healthy brain tissue.

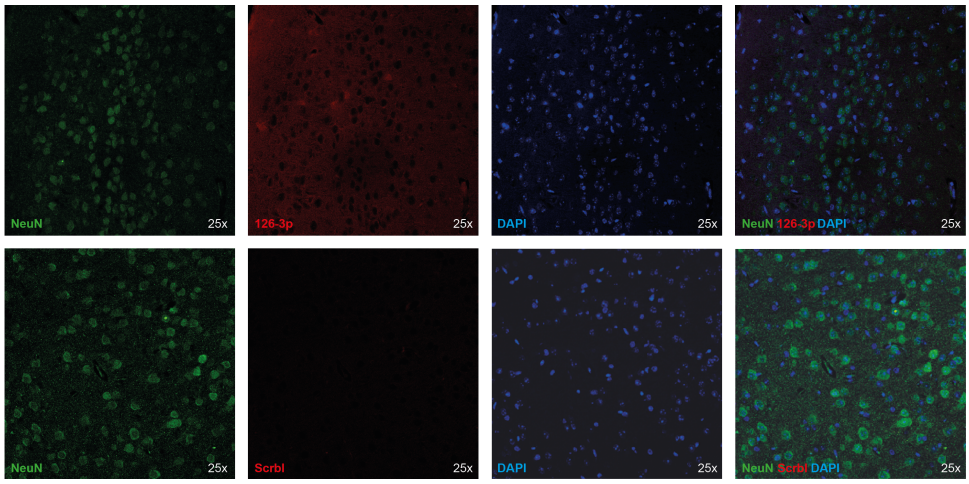

Figure 17: miR-126-3p + neuron staining in healthy murine brain tissue
Alexa Flour 488 staining of NeuN-labeled neurons, Cy3 staining of digoxigenin-labeled miR-126-3p and scramble miRNA as negative control, respectively, DAPI staining of nucleoli and merge in healthy murine brain tissue. Scrbl = Scramble miRNA probe.

When staining astrocytes with GFAP instead, no colocalization with areas of enhanced miR-126-3p expression could be detected either.

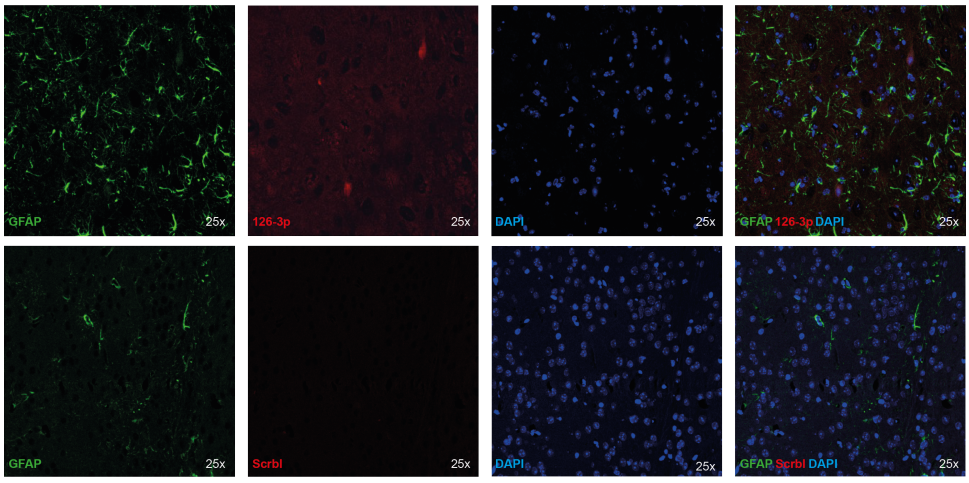

Figure 18: miR-126-3p + astrocyte staining in healthy murine brain tissue
Alexa Flour 488 staining of GFAP-labeled astrocytes, Cy3 staining of digoxigenin-labeled miR-126-3p and Scramble miRNA as negative control, respectively, DAPI staining of nucleoli and merge in healthy murine brain tissue. Scrbl = Scramble miRNA probe.

In the murine stroke core area, stains showed scattered nucleoli and destroyed

neurons or astrocytes, respectively, alongside several remaining cells and fragments. miR-126-3p showed unspecific enhancement with no association to degraded cells.

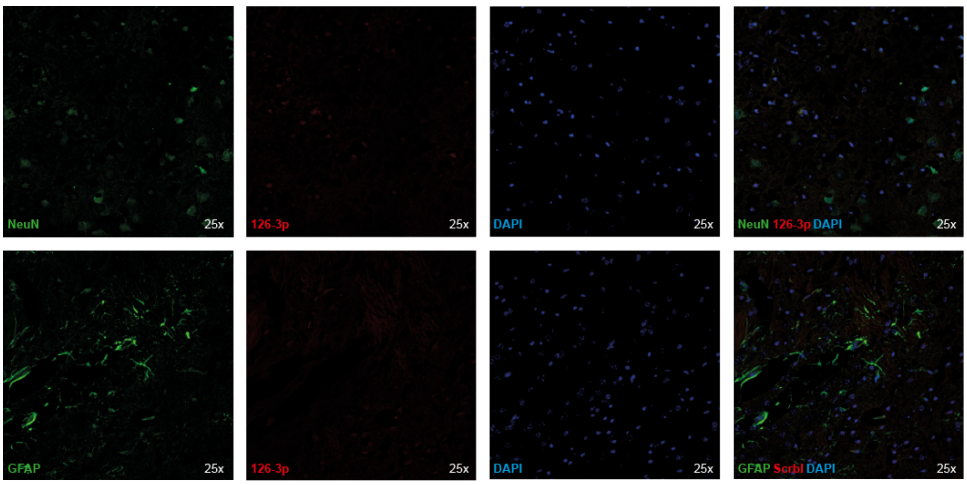

Figure 19: miR-126-3p + neuron and astrocyte staining in murine stroke core area Alexa Flour 488 staining of NeuN-labeled neurons and GFAP-labeled astrocytes, respectively, Cy3 staining of digoxigenin-labeled miR-126-3p, DAPI staining of nucleoli and merge in murine stroke core area. Scrbl = Scramble miRNA probe.

5 Discussion

Our study identifies a set of five circulating platelet-associated miRNAs, which show differential expression in the acute phase after ischemic stroke. miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p and miR-223-3p showed increased plasma levels in both derivation and replication sample in ischemic stroke patients compared to healthy controls. miR-126-3p and miR-223-3p also showed significantly elevated plasma levels in comparison to plasma levels in TIA patients.

A longitudinal analysis of those miRNAs on days 2, 3, 7 and 90 after stroke showed that plasma levels quickly returned to baseline levels on day 2 after stroke and did not show any significant changes afterwards, with the exception of miR-24-3p, whose levels remained significantly elevated until day 3. In terms of discriminating between ischemic stroke and healthy controls, a combination of these miRNAs showed an AUC of 0.82, translating into a sensitivity of 84% and a specificity of 68%, which is higher than the sensitivity and specificity of CT scans in the early hours after an ischemic stroke [128]. When differentiating between ischemic stroke patients and TIA patients, the AUC was 0.57, which corresponds to a sensitivity of 39% and specificity of 82%. After analyzing the AUC of our platelet-associated miRNAs, we combined the five miRNAs that we identified in our previous publication [65], with the five miRNAs of the platelet-association study. This resulted in a total AUC of 0.94 (sensitivity 82%, specificity 92%).

All five miRNAs also showed increasing plasma levels with the number of platelets added in a platelet spike-in experiment. When comparing ischemic stroke patients and healthy controls who had been taking ASA in the previous seven days, miR-21-5p and miR-223-5p did not show significant changes in plasma levels in the ischemic stroke group anymore, suggesting an impact of platelets on miRNA plasma levels.

When analyzing differences in working speed and impact of differential centrifugation steps, minor changes in protocols of blood sampling and RNA isolation did not lead to significantly altered miRNA yield or results. However, as suggested by the platelet spike-in experiment, platelet numbers in the plasma samples had a significant impact on miRNA levels.

In the experimental murine photothrombosis model, none of the five miRNAs showed a significant increase or directionality in line with the findings in human ischemic stroke patients. Combined FISH and immunofluorescent labeling did

not show a colocalization of miR-126-3p expression with neurons or astrocytes in the murine brain.

Diagnostic utility of platelet-associated miRNAs in a clinical setting

To our knowledge, none of the blood-based biomarkers assessed for their diagnostic utility have surpassed the receiver operating characteristics of the identified miRNAs so far. Blood-based proteins such as GFAP, S100B or NSE lack sensitivity especially in the early phase of ischemic stroke, since they peak on day 2 after ischemic stroke, or later [60, 61, 63]. Recent studies have identified miRNAs that show specific expression patterns and elevation in plasma levels in the first hour after myocardial infarction [129, 130], stressing the potential of miRNAs as diagnostic biomarkers in the acute phase.

Currently, CT imaging is the first choice in diagnostics of acute ischemic stroke [131]. However, since CT scans only show a sensitivity of about 60% [128], the miRNAs identified in our study, who showed a sensitivity of 84% in the acute phase, may have diagnostic potential for early ischemic strokes in the future. In order to replace or support neuroimaging as the primary diagnostic measure in stroke diagnostics, it would be essential to find an assay that is able to quantify miRNA levels within a few minutes in order to contribute to acute decision making in the initial stroke phase to the same extent as CT scans currently do. So far, the mean time from blood draw to retrieving miRNA level results from qPCR analysis is 5-6 hours, an issue that needs to be resolved in order to approach CT's usefulness in acute diagnostics.

In order to ensure specificity, further studies might address whether a similar miRNA expression profile is present in other neurological diseases such as intracranial hemorrhages, seizures, migraines or head trauma, which pose important differential diagnoses in setting of a patient with sudden onset of neurological symptoms. In addition, the differentiation between ischemic and hemorrhagic stroke is of crucial importance for a diagnostic biomarker used in the acute clinical setting. Some miRNAs have been identified to show significantly elevated plasma levels in hemorrhagic stroke patients compared to healthy controls [132]. If, in the future, further experiments show that our miRNAs show a similar expression pattern in hemorrhagic stroke as they do in ischemic stroke, adding more miRNAs to the analysis may be an option. Since the miRNAs identified in our experiment are linked to platelet activation or aggregation, it is possible that these miRNAs show a similar elevation of plasma levels in

other pathologies involving thrombus or embolus formation, such as myocardial infarctions, pulmonary embolisms or deep vein thrombosis. However, since these patients can usually be distinguished from stroke patients by thorough clinical examination only, this might not necessarily restrict the potential of the miRNAs. A disease that might, in some cases, be confounded with ischemic stroke patients by clinical presentation could be cerebral vein thrombosis, and miRNA plasma levels of those patients are yet to be examined.

In our experiments, miR-126-3p and miR-223-3p were shown to be significantly altered in ischemic stroke patients in comparison to TIA patients. Being able to distinguish an ischemic stroke patient from a TIA patient would remarkably influence the clinical decision for or against systemic lysis therapy. However, the sensitivity of 39% does not suffice in order to serve as a diagnostic biomarker for this specific clinical challenge. So far, to our knowledge, other attempts to find a biomarker that could successfully differentiate between TIA and ischemic stroke were also unsuccessful [133].

Four of the five miRNAs identified in our study showed elevated plasma levels after ischemic stroke that quickly returned to baseline levels on day 2 after stroke. In the past few years, many studies have focused on treatment of stroke patients with unknown onset time of symptoms, such as wake-up strokes. Further analyses of the exact times of rise and decrease of circulating miRNAs on day 1 after stroke might reveal a specific time course that might enable us to draw conclusions on the time of stroke onset retrospectively. Being able to estimate the time of stroke onset and thus determining whether a patient is eligible for intravenous thrombolysis would remarkably improve treatment options of ischemic stroke patients with unknown symptom onset.

Recently, numerous publications have focused on plasma levels of miRNAs, but since every laboratory uses different procedures, working conditions and protocols, it still needs to be shown to what extent results from different laboratories are comparable and replicable, and how much of the changes in miRNA levels in human plasma can be traced back to working conditions, cell degradation or cell loss. When analyzing differences in working speed, differential centrifugation steps and number of platelets present in plasma samples during RNA isolation, plasma levels in platelet-rich plasma were significantly higher than in platelet-poor plasma in miR-126-3p, miR-126-5p and miR-223-3p. No significant differences could be observed for miR-122-5p. This is in line with our previous experiments and further emphasizes the fact that in studies

researching cardiovascular or cerebrovascular pathologies, the contribution of platelets to circulating miRNA levels needs to be carefully considered in order to avoid confounding of results. None of the miRNAs examined showed significant differences in results after an extra centrifugation step or after different incubation times immediately after blood draw. This suggests potential for the miRNAs to serve as reliable biomarkers, even when measured in different settings and laboratories. However, standardized experimental conditions, equipment and cut-off levels would be required prior to establishing miRNAs as blood-based biomarkers in a clinical setting. Possibly confounding or complicating factors that were not addressed in our experiments may include hemolysis during blood draw or remainder of subcellular or cellular components in the samples [134].

The results of the longitudinal analysis suggest that the release of miRNAs into the circulation after ischemic stroke is linked to the acute stage and does not seem to be influenced by later molecular processes in stroke such as cell damage, tissue reorganization or inflammation. Further analyses of processes of secretion, release and regulation of platelet-associated miRNAs will be essential in order to determine their potential in diagnostics.

Platelets: a major source of circulating miRNAs

Underlying molecular processes of regulation, secretion and cellular sources of the miRNAs identified in setting of ischemic stroke are yet to be discovered. As a first approach, we identified platelets as major sources of all five miRNAs in our experiments.

In the platelet spike-in experiment, plasma levels of all five miRNAs depended on the number of platelets spiked in. These findings are in line with those of Kaudewitz et. al. in 2016, who discovered miR-223-3p and miR-126-3p to be dependent on platelet numbers in a platelet spike-in experiment. This study also revealed that miR-126-3p and miR-223-3p, and, to a lesser extent, miR-21-5p and miR-24-3p 'correlated with plasma levels of P-selectin, platelet factor 4 and platelet basic protein in the population-based Bruneck study (n=669)' [113]. These results, in combination with our findings in ischemic stroke patients, suggest a strong link to platelet function for the miRNAs identified and underline the importance of further investigating molecular mechanisms not only in ischemic stroke, but in other pathologies involving platelet aggregation and thrombosis.

In the ischemic stroke patient group, negative control miR-122-5p did not

show significant changes compared to the control group, nor did miR-150-5p, which has been shown not to be associated with the number of platelets added in the platelet spike-in experiment. Those findings further support the hypothesis that the elevated plasma levels of platelet-associated miRNAs might occur due to an association with thrombus formation, platelet aggregation or activation processes in ischemic stroke pathophysiology. However, these data do not enable us to draw conclusions about causality: It is possible that the five miRNAs are, in fact, actively secreted or released from platelets during activation. Nonetheless, it is also possible that plasma levels of these miRNAs had been elevated prior to the stroke, due to increased platelet activation status in patients with future ischemic stroke, or that increased plasma levels of platelet-associated miRNAs might even be associated with a higher risk for ischemic stroke. Analyzing whether there is a correlation between the platelet-associated miRNAs identified and thrombus size and clot burden in the ischemic stroke group could be a possible next step towards investigating the role of the elevated miRNA plasma levels in the acute stroke setting. The same applies for correlation for infarct volumes, but might be of limited importance for a diagnostic marker for ischemic stroke, as small strokes in eloquent areas can cause more damage to the patient than a large stroke in a different part of the brain [54].

When our experiments revealed that platelets are a major cell type contributing to circulating plasma miRNA levels in ischemic stroke patients, the question was raised whether prior antiplatelet treatment, which is common especially in patients with cardiovascular risk factors and therefore also in the study cohort, has an influence on miRNA levels in those subjects. When comparing miRNA plasma levels in ischemic stroke patients and healthy controls who all had been taking ASA in the week prior to the blood draw, no significant elevation of miR-21-5p and miR-223-3p plasma levels could be observed, in contrast to the group without ASA treatment. Plasma levels of miR-24-3p, miR-126-3p and miR-126-5p were still significantly elevated but showed a more discrete elevation than in the original experiment.

These findings were in line with the identification of prior antiplatelet therapy as a relevant covariate in the replication sample, revealing that antiplatelet therapy might affect the release of platelet-associated miRNAs, and that results obtained in experiments that involve platelet-linked miRNAs need to be adjusted by multivariate analysis.

The effect of antiplatelet therapy on circulating miRNA levels has previously

been addressed in several studies. In 2013, Willeit et al. [106] analyzed 8 miRNAs, which we also assessed in our experiments, in 9 healthy volunteers and compared miRNA levels after no treatment, or treatment with 10mg prasugrel, 10mg prasugrel + 75mg ASA or 10mg prasugrel + 300mg ASA. In this first experiment, miR-21-5p, miR-24-3p, miR-126-3p and miR-223-3p showed a negative correlation with increasing dosage of antiplatelet therapy. Interestingly, a negative correlation with antiplatelet therapy could also be detected in miR-20b-5p, miR-150-5p, miR-191-5p and miR-197-3p, all of which are miRNAs that we had excluded because of lacking correlation with platelet numbers in the platelet spike-in experiment or because the concentration in plasma samples of healthy subjects was not sufficient to be reliably detected in the plasma samples used in our experiments. This suggests that other miRNAs such as miR-150-5p, even though not directly involved in platelet function, play an important role in mechanisms leading to or following platelet aggregation or platelet activation and might also be related to the pathophysiological processes in ischemic stroke and other cardiovascular pathologies, which yet needs to be investigated.

De Boer et al. confirmed in an *in vitro* experiment that miR-126-3p is released into plasma upon platelet activation, and that plasma levels of this miRNA correlate with platelet activation in diabetes mellitus type 2 patients. When administering ASA, circulating miR-126-3p levels were significantly lower in comparison to a placebo treatment [135].

Results of these studies implicate that the effects of antiplatelet drugs on circulating plasma miRNA levels are a crucial factor in the assessment of miRNAs in cardiovascular and cerebrovascular diseases and must be taken into account in future studies and experiments.

Plasma levels and cellular sources of miRNAs in murine stroke models

When our experiments showed that platelets are a major contributor of circulating miRNAs in blood plasma in humans, we became interested in discovering whether we could reproduce those findings in murine models, which could be a step towards further investigating molecular and pathophysiological mechanisms in the future. There is a variety of experimental murine stroke models, which all intend to reflect different types or different pathophysiological processes of human stroke. For our experiments, we analyzed the miRNAs selected for our study in the photothrombosis model, which involves platelet aggregation and thrombus formation induced by a laser. In this experiment, none of the five

miRNAs that are differentially expressed in human ischemic stroke showed significant alterations in the photothrombosis stroke model, compared to a sham group. The only exception to this was miR-21-5p, which was significantly decreased in the stroke model compared to the sham group, contradicting the increase in plasma levels in human ischemic stroke patients. In addition, plasma levels of miR-30b-5p and miR-150-5p were significantly decreased in this model, contradicting the results in the human experiment as well.

To our knowledge, there are no similar studies that assess the expression of miRNAs in a murine photothrombosis stroke model. However, there are some studies that use middle cerebral artery occlusion (MCAo) models to assess alterations in miRNA expression levels: in 2008, Jeyaseelan et al. [136] identified aberrantly expressed miRNAs in rat blood and brains after transient ischemia was induced by MCAo, which included miR-150-5p and miR-191-5p. Both miRNAs were upregulated at both 24 and 48 hours after induced stroke, however, since we analyzed murine miRNA plasma levels at 6 hours after induced stroke, those findings are of limited comparability. Dharap et al. [137] identified several miRNAs that are up- or downregulated at 3, 6, 12, 24 and 72h after MCAo, but none of the miRNAs that were differentially expressed had been part of our experiments.

We were not able to find a miRNA that showed consistent changes and directionality in human ischemic stroke patients and mice with induced experimental stroke by the photothrombosis model. One reason for this could be traced back to slightly different nucleotide sequences in human and murine miRNAs, as well as differences in posttranscriptional regulations in both species [125]. As a second reason, all experimental murine stroke models can only partially mimic the pathophysiological events that occur in a human ischemic stroke, and it is possible that the processes in ischemic stroke patients that lead to platelet aggregation, thrombus formation and miRNA release are not properly reflected by the models we used in our experiments [138]. Experiments done in a different species are always limited, because of substantial differences in physiology and pathophysiology inbetween different species, which cannot always be overcome by drafting an experimental animal model.

However, further studying platelet-linked miRNAs and pathophysiological processes as well as further improving experimental murine stroke models may eventually lead to the identification of miRNAs whose function can successfully be reflected in murine stroke models. Studies done on miRNAs in acute my-

ocardial infarction are a good example: miR-208a and miR-499 have been found to be elevated in acute myocardial infarctions and have potential as diagnostic biomarkers [99, 105]. Xiao et al. showed that plasma levels of both miRNAs are accordingly elevated in mice with induced myocardial infarction by coronary artery ligation after 4 and 24 hours [139].

When combining FISH of specific cell types with immunofluorescent labeling of miRNAs, no colocalization of miR-126-3p expression with neurons or astrocytes could be observed neither in healthy brain tissue, nor in the infarct core area of mice with induced experimental stroke. This may suggest that plasma levels of those miRNAs are mainly linked to platelet function and aggregation and not be influenced by active or passive secretion by brain cells. However, in contrast, a study performed on rat brain sections showed that miR-21-5p expression is upregulated in neurons in the ischemic area [140]. Furthermore, recent studies found miR-126-3p to be expressed in neurons, and involved in their function in humans [141, 142], miR-223-3p levels are elevated in neurons in autoimmune encephalomyelitis [143] and miR-21-5p has been found to be expressed in oligodendrocytes [144]. Thus, future experiments will require optimization of experimental conditions and the identification of accurate incubation temperatures and times to ensure specific staining and labeling of cells and miRNAs, in order to further examine cell-specific expression in the human and murine brain.

In order to find a murine stroke model that appropriately reflects pathophysiological processes in human ischemic stroke as well as changes in plasma levels of platelet-associated miRNAs, further investigations of pathophysiology by using microRNA-antagonists (antagomiRs) and by extracting platelets or brain cells from murine stroke models might be necessary. Another approach could be to induce a stroke in ApoE-deficient mice in order to improve reflection of the atherosclerotic and endothelial processes leading to a stroke in humans.

Pathophysiological processes in ischemic stroke involving platelet-linked miRNAs

Besides their occurrence in platelets, the miRNAs identified are involved in biological processes in the blood vessels as well as in pathophysiological processes in ischemic stroke. A previous study showed that 'miR-21 overexpression reduced endothelial cell proliferation, migration and the ability of these cells to form tubes' [145]. In line with our findings, miR-21-5p and miR-24-3p

have been described as potential diagnostic biomarkers for ischemic stroke and atherosclerosis in two recent publications [146, 147]. miR-24-3p has been shown to be involved in angiogenesis as well [148], while miR-126-3p 'mediates developmental angiogenesis in vivo' [149]. It has also been shown to be atheroprotective [150]. Levels of this miRNA correlate with the severity of cerebral atherosclerosis [151], and 'with lower disease risk, decreased disease severity, and reduced inflammatory cytokines in AIS [(acute ischemic stroke)] patients' [152]. Underlining the association to platelets described in our experiments, both miR-126-3p and miR-223-3p are involved in regulation of platelet reactivity and function in diabetes mellitus patients [111]. miR-126-3p and miR-126-5p are two of the most abundantly expressed miRNAs in endothelial cells [150]. Schober et al. found that miR-126-5p prevents the formation of atherosclerotic lesions and is substantial to endothelial repair. In addition, his group discovered that miR-126-5p expression in endothelial cells is induced by vascular shear stress [89]. miR-223-3p has been widely described and linked to platelet function and aggregation in recent literature: it targets the $P2Y_{12}$-receptor [123] on platelets which plays a major role in the process of platelet aggregation [153]. Mice with antagonized miR-223-3p form larger thrombi in comparison to wild type mice [111], and miR-223-3p influences response to clopidogrel [124]. In an analysis of 50 human ischemic stroke patients, Chen et al. showed that 'increased circulating exosomal miR-223 is associated with acute ischemic stroke occurrence, stroke severity, and short-term outcome' [154].

Taking all of these findings into account, we cannot know with absolute certainty whether altered miRNA plasma expression profiles are directly involved in platelet activation or aggregation, and if alterations are cause or result of platelet function. Furthermore, it is possible that the miRNAs indirectly affect platelet function by being involved in endothelial processes which lead to platelet aggregation. Considering the complexity of the pathophysiology leading to atherosclerosis, platelet activation as well as to ischemic stroke and other cardiovascular pathologies, the exact role of miRNAs remains unclear and further investigation is required in the future.

Strengths and limitations

To our knowledge, our study presents the analysis of miRNA levels after ischemic stroke with the largest patient number in current literature. Overall, our study included almost 500 patients acquired over the time span of three years. Significant elevation of all miRNAs in the derivation sample after ischemic stroke

could be replicated in the replication sample. Not only did we analyze expression levels in comparison to healthy controls, but we also included TIA patients, since reliably diagnosing a TIA and distinguishing it from an ischemic stroke is an essential part in the decision making for the most appropriate therapy. In our analysis, we thoroughly ruled out confounding factors: In the derivation cohort, we excluded patients with prior treatment with antiplatelet drugs or heparin, recent cardiovascular events, signs for silent CNS infarctions and recent major diseases or surgeries. In both derivation and replication sample, we carefully identified relevant covariates such as demographic factors and vascular risk factors, which we accounted for in a multivariate statistical model. None of our study subjects had been treated with heparin in the weeks prior to the blood draw, thus interference of heparin with the qPCR enzymes was avoided. Upon hospital arrival, blood of the ischemic stroke and TIA patients was immediately drawn, prior to starting diagnostic and therapeutic measures, which allowed us to analyze miRNA plasma levels in the very early hours after symptom onset. Involving ischemic stroke patients and their relatives as healthy controls in frequent follow-up exams at the outpatient clinic enabled us to follow up on miRNA expression and the dynamics up to 90 days after stroke. All experiments were performed by adding a 'normalization control [that] is stably expressed and not affected by experimental conditions or clinical parameters' [108]. In our experiments, synthetic intrinsic spike-in controls such as UniSp4 and UniSp6 were used, as well as normalization to average C_t-values of all analyzed miRNAs [155]. All of our experiments were performed using a double randomization: Ischemic stroke patients, TIA patients and healthy control subjects were analyzed in randomly assigned groups on cDNA and qPCR plates. During the entire experiment, the patient identification remained unknown to the experimenter, as well as the origin (stroke, TIA or control) of the sample.

Our experiments also have limitations. All of our study subjects were recruited from one single hospital, which might lead to selection bias of the study group. As only a part of the initial patients returned for the follow-up exam on day 90 after stroke, it cannot be excluded that some patients did not return due to a bad outcome or severe disability, thus biasing the results. We therefore cannot exclude that miRNA levels on day 90 are different in patients with large and severe infarctions. In addition, miRNAs are sensitive to degradation during RNA-isolation, reverse transcription and qPCR, and might have been influenced by differing experimental conditions such as waiting times or room temperature [115]. By performing several pre-tests prior to analyzing samples of the study cohorts, we standardized experimental procedures and protocols and identified

possible confounding variates. When analyzing the replication sample or the longitudinal analysis, the number of study subjects, and hence samples, was too large to fit on one single qPCR plate. In order to avoid the results being biased by this, we added recombinant miRNA samples with determined miRNA copy numbers on each plate and normalized the results to changes that might have influenced the results during qPCR. Even though our results showed significantly elevated miRNA plasma levels in the early hours after symptom onset, we cannot exclude that those levels had been elevated prior to the stroke. However, since four of the five miRNAs showed plasma levels that quickly returned to normal on day 2, it is likely that the changes in miRNA levels are related to the acute event of stroke onset.

Conclusion

In conclusion, circulating plasma levels of platelet-associated miRNAs are significantly elevated in ischemic stroke patients and healthy controls. Overall, we consider these miRNAs to have potential as diagnostic biomarkers for acute ischemic stroke in the future.

6 Summary

Stroke is one of the leading causes of death and disability worldwide and requires fast diagnosis and therapy in order to minimize permanent brain damage. So far, neuroimaging is the best and most accurate way to diagnose an ischemic stroke, and CT scans can rule out a hemorrhagic stroke with high sensitivity. However, CT lacks sensitivity in distinguishing an ischemic stroke from stroke mimics such as seizures, migraines or cerebral infections in the early hours after stroke onset. There are recent approaches to find a diagnostic blood-based biomarker for ischemic stroke as well as for transient ischemic attack (TIA), such as neuron-specific enolase (NSE), S100B or glial fibrillary acidic protein (GFAP), or coagulation or inflammatory markers, but their potential as diagnostic biomarkers is limited due to lacking sensitivity or a delayed response after symptom onset.

microRNAs (miRNAs) are small, non-coding RNAs, that can circulate in the blood with high stability and cell- and tissue-specific expression patterns. Platelet aggregation and thrombus formation are essential pathophysiological processes in both embolic and thrombotic ischemic stroke. For many miRNAs, platelets have been identified as a major source. Thus, the goal of this dissertation was to analyze plasma levels of platelet-associated miRNAs in ischemic stroke and TIA patients, to assess their potential as diagnostic biomarkers and to investigate molecular mechanisms of miRNA release and cellular sources in murine stroke models.

First, we selected miRNAs that had been described to be associated with platelets in a systemic literature search. We quantified plasma levels of those miRNAs in a healthy human volunteer and selected eight miRNAs for subsequent experiments. In a platelet spike-in experiment, we compared miRNA plasma levels in platelet-poor plasma (PPP) with different numbers of platelets spiked in to platelet-rich plasma (PRP), and identified seven miRNAs whose plasma levels depended on the number of platelets spiked in. Next, we compared plasma levels of the miRNAs in 60 ischemic stroke patients to 60 healthy controls in the derivation sample. The five miRNAs who showed significantly elevated plasma levels in the ischemic stroke group (miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p and miR-223-3p) also showed significantly elevated plasma levels in the replication sample, which included 200 ischemic stroke patients and 100 healthy controls. miR-126-3p and miR-223-3p also showed significantly elevated plasma levels in ischemic stroke patients compared to TIA patients. A longitudinal analysis showed that all miRNAs returned to baseline levels on day

2 after stroke, with the exception of miR-24-3p, whose plasma levels remained elevated up to day 3. miR-21-5p and miR-223-3p did not show elevated plasma levels in a group of ischemic stroke patients compared to healthy controls, all of which had been treated with acetylsalicylic acid (ASA) before, suggesting a platelet aggregation link to miRNA release. A receiver operating characteristics analysis of the five miRNAs resulted in a combined sensitivity of 84%, which is higher than the sensitivity of CT imaging in the early hours after symptom onset.

Plasma levels of the platelet-associated miRNAs did not show similar significant changes or directionality in a murine experimental stroke model including photothrombosis. Combined fluorescent in-situ hybridization and immunofluorescent labeling did not show a colocalization of miR-126-3p and neurons or astrocytes, respectively.

Overall, we suggest that platelet-associated miRNAs are potential diagnostic biomarkers for ischemic stroke in the future.

7 Zusammenfassung

Der Schlaganfall ist eine der häufigsten Todesursachen weltweit sowie eine der führenden Ursachen für eine bleibende Behinderung. Um bleibende Gehirnschäden zu verhindern, sind eine schnelle Diagnose und Therapie essenziell. Aktuell sind bildgebende Verfahren die beste und genaueste Methode, um einen ischämischen Schlaganfall zu diagnostizieren. Die CT-Bildgebung kann einen hämorrhagischen Schlaganfall mit hoher Sensitivität ausschließen. Bei der differentialdiagnostischen Abgrenzung eines ischämischen Schlaganfalls von sogenannten 'Stroke mimics' wie Krampfanfällen, Migräneattacken oder zerebralen Infektionen ist die Sensitivität jedoch deutlich geringer, besonders in den ersten Stunden nach Auftreten der Symptomatik. In letzter Zeit beschäftigten sich zahlreiche Studien mit der Suche nach einem Biomarker, sowohl für den ischämischen Schlaganfall als auch für die transiente ischämische Attacke (TIA) im Blut. Zahlreiche neuronale und gliale Marker wie die neuronenspezifische Enolase (NSE), S100B oder saures Gliafaserprotein (GFAP), Gerinnungs- sowie Entzündungsmarker wurden untersucht, zeigten jedoch aufgrund geringerer Sensitivität sowie verspäteter Antwort nach Auftreten der Symptomatik nur ein eingeschränktes Potential als diagnostischer Biomarker.

microRNAs (miRNAs) sind kleine, nicht-kodierende RNAs, die im Blut mit hoher Stabilität zirkulieren, sowie zell- und gewebespezifische Expressionsmuster zeigen. Thrombozytenaggregation und Thrombusbildung sind essenzielle pathophysiologische Prozesse sowohl beim embolischen als auch beim thrombotischen ischämischen Schlaganfall. Da Thrombozyten in der Literatur als Quelle zahlreicher miRNAs identifiziert wurden, war das Ziel der Dissertation 1. die Untersuchung der Plasmakonzentration von mit Thrombozyten assoziierten miRNAs in Patienten mit ischämischem Schlaganfall und TIA verglichen mit Kontrollen, 2. ihr Potential als diagnostische Biomarker zu analysieren und 3. in Mausmodellen die molekularen Mechanismen und den zellulären Ursprung der miRNAs zu untersuchen.

Zuerst wurden in einer systemischen Literaturrecherche miRNAs ausgewählt, die mit Thrombozyten oder deren Funktion assoziiert sind. Die Konzentrationen dieser miRNAs wurden zunächst an einem gesunden Probanden untersucht. Anschließend wurden acht miRNAs mit zuverlässig nachweisbaren Plasmakonzentrationen für die weiteren Experimente ausgewählt. In einem sogenannten 'Platelet Spike-in Experiment' wurde die Plasmakonzentration in thrombozytenfreiem Plasma, zu dem verschiedene Konzentrationen von

Thrombozyten hinzugefügt wurden, mit thrombozytenreichem Plasma verglichen. Es konnten sieben miRNAs identifiziert werden, deren Konzentration mit zunehmender Thrombozytenanzahl in der Probe korrelierte.

Danach wurde die Plasmakonzentration dieser miRNAs in 60 Patienten mit ischämischem Schlaganfall im Vergleich zu 60 gesunden Kontrollprobanden analysiert. Die Erhöhung der Konzentration von fünf miRNAs (miR-21-5p, miR-24-3p, miR-126-3p, miR-126-5p und miR-223-3p) in der Schlaganfall-Gruppe konnte in einer weiteren Versuchsgruppe mit 200 Schlaganfallpatienten und 100 Kontrollen bestätigt werden. In dieser Gruppe zeigte sich auch eine signifikante Erhöhung von miR-126-3p und miR-223-3p in Schlaganfallpatienten im Vergleich zu Patienten mit einer TIA. Eine longitudinale Analyse zeigte, dass die Konzentration von vier der fünf miRNAs schon am Tag 2 nach dem ischämischen Schlaganfall wieder zu normalen Plasmaspiegeln zurückkehrte, nur miR-24-3p zeigte noch eine signifikant erhöhte Plasmakonzentration bis zu Tag 3 nach Auftreten der ersten Symptome. In einer Versuchsgruppe, in der alle Teilnehmer zuvor mit Acetylsalicylsäure (ASS) behandelt worden waren, war die Konzentration von miR-21-5p und miR-223-3p in der ischämischen Schlaganfallgruppe im Vergleich zur gesunden Kontrollgruppe nicht mehr erhöht, was auf einen starken Zusammenhang der Thrombozytenaggregation mit einer erhöhten Expression der miRNAs schließen lässt.

Eine ROC-Analyse ergab bei Kombination der fünf miRNAs bei Vergleich von ischämischem Schlaganfall zu gesunden Kontrollen eine Sensitivität von 84%. Diese ist höher als die Sensitivität der CT-Bildgebung in den ersten Stunden nach Auftreten des Schlaganfalls. Im experimentellen Mausmodell zeigten die miRNAs keine vergleichbaren Veränderungen oder Direktionalitäten wie bei humanen Schlaganfallpatienten. In einer kombinierten Fluoreszenz-Hybridisierung mit immunofluoreszenter Markierung von Zellen zeigte sich keine Kolokalisation der Areale mit erhöhter miR-126-3p-Expression mit Neuronen oder Astrozyten.

Zusammenfassend haben thrombozytenassoziierte miRNAs großes Potential als diagnostische Biomarker für den ischämischen Schlaganfall.

8 List of Abbreviations

ADP	adenosine diphosphate
AGO2	Argonaute 2
antagomiR	microRNA-antagonists
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
AUC	area under the curve
cDNA	complementary DNA
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computed tomography
C_t-value	threshold cycle value
D/C	discharge
DAPI	4',6-Diamidino-2-Phenylindole
DEPC	Diethylpyrocarbonate
dMCAo	distal middle cerebral artery occlusion
dNTPs	deoxyribonuclease triphosphates
DWI	diffusion-weighted imaging
ECG	electrocardiography
ED	emergency department
EDC	ethylcarbodiimide
FISH	fluorescent in-situ hybridization
FLAIR	fluid-attenuated inversion recovery
GFAP	glial fibrillary acidic protein
ICAM	intercellular adhesion molecule
IL-6	interleukin-6
ISD	Institute for Stroke and Dementia Research
MACS	magnetic activated cell sorting
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MI	myocardial infarction
miR	microRNA

miRNA	microRNA
MRI	magnetic resonance imaging
mRNA	messenger RNA
NfL	Neurofilament light protein
NGS	normal goat serum
NSE	Neuron-specific enolase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPP	platelet-poor plasma
Pre-miRNA	precursor microRNA
Pri-MIRNA	primary microRNA
PRP	platelet-rich plasma
PT	photothrombosis
RISC	RNA-induced silencing complex
ROC	receiver operating characteristics
RT	reverse transcription
RT-qPCR	reverse transcription quantitative polymerase chain reaction
Scrbl	scramble miRNA probe
SD	standard deviation
SEM	standard error of the mean
SMC	smooth muscle cell
SSC	saline sodium citrate
TBS	Tris-buffered saline
TGF-β	transforming growth factor beta
TIA	transient ischemic attack
TNFα	tumor necrosis factor alpha
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	tissue plasminogen activator
UTR	untranslated region
VCAM	vascular cell adhesion molecule

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References

[1] R. Lozano, M. Naghavi, K. Foreman *et al.*, “Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010,” *Lancet*, vol. 380(9859), pp. 2095–128, 2012.

[2] A. Bakhai, “The Burden of Coronary, Cerebrovascular and Peripheral Arterial Disease,” *PharmacoEconomics*, vol. 22, pp. Suppl. 4:11–18, 2004.

[3] W. Rosamond, K. Flegal, G. Friday *et al.*, “Heart Disease and Stroke Statistics - 2007 Update,” *Circulation*, vol. 115, pp. e69–171, 2007.

[4] C. Eyileten, Z. Wicik, S. De Rosa, D. Mirowska-Guzel, A. Soplinska, C. Indolfi, I. Jastrzebska-Kurkowska, A. Czlonkowska, and M. Postula, “MicroRNAs as Diagnostic and Prognostic Biomarkers in Ischemic Stroke—A Comprehensive Review and Bioinformatic Analysis,” *Cells*, vol. 7(12), p. 249, 2018.

[5] B. Ovbiagele, “National Sex-specific Trends in Hospital-Based Stroke Rates,” *Journal of Stroke and Cerebrovascular Diseases*, vol. 20,6, pp. 537–540, 2011.

[6] K. Strong, C. Mathers, and R. Bonita, “Preventing stroke: saving lives around the world,” *Lancet Neurology*, vol. 6, pp. 182–87, 2007.

[7] D. Lloyd-Jones, R. Adams, T. Brown *et al.*, “Heart Disease and Stroke Statistics - 2010 update: a report from the American Heart Association,” *Circulation*, vol. 121, pp. e46–e215, 2010.

[8] A. El-Saed, L. Kuller, A. Newman, O. Lopez, J. Costatino, K. McTigue, M. Cushman, and R. Kronmal, “Geographic variations in stroke incidence and mortality among older populations in four US communities,” *Stroke*, vol. 37, pp. 1975–1979, 2006.

[9] A. Boehme, C. Esenwa, and M. Elkind, “Stroke Risk Factors, Genetics, and Prevention,” *Circulation Research*, vol. 120(3), pp. 472–495, 2017.

[10] M. O’Donnell, S. Chin, S. Rangarajan *et al.*, “Global and regional effects of potentially modifiable risk factors associated with acute stroke in 32 countries (INTERSTROKE): a case-control study,” *Lancet*, vol. 388, pp. 761–75, 2016.

[11] J. Chong and R. Sacco, "Risk Factors for Stroke, Assessing Risk, and the Mass and High-Risk Approaches for Stroke Prevention," *CONTINUUM Lifelong Learning in Neurology*, vol. 11, pp. 18–34, 2005.

[12] P. Wolf, R. D'Agostino, A. Belanger, and W. Kannel, "Probability of stroke: a risk profile from the Framingham study," *Stroke*, vol. 22, pp. 312–318, 1991.

[13] A. Folsom, M. Rasmussen, L. Chambless, G. Howard, L. Cooper, M. Schmidt, and G. Heiss, "Prospective associations of fasting insulin, body fat distribution, and diabetes with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators," *Diabetes Care*, vol. 22(7), pp. 1077–1983, 1999.

[14] V. Roger, A. Go, D. Lloyd-Jones *et al.*, "Heart Disease and Stroke Statistics - 2011 Update," *Circulation*, vol. 123(4), pp. e18–e209, 2011.

[15] P. Deb, S. Sharma, and K. Hassan, "Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis," *Pathophysiology*, vol. 17, pp. 197–218, 2010.

[16] H. Adams, B. Bendixen, L. Kappelle, J. Biller, B. Love, D. Gordon, and E. Marsh, "Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment," *Stroke*, vol. 24, pp. 35–41, 1993.

[17] H. Stary, "Macrophages, macrophage foam cells, and eccentric intimal thickening in the coronary arteries of young children," *Atherosclerosis*, vol. 64, pp. 91–108, 1987.

[18] J. Ip, V. Fuster, L. Badimon, J. Badimon, M. Taubman, and J. Chesebro, "Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation," *Journal of the American College Cardiology*, no. 15, pp. 1667–1678, 1990.

[19] C. Slager, J. Wentzel, F. Gijsen, A. Thury, A. van der Wal, J. Schaar, and P. Serruys, "The role of shear stress in destabilization of vulnerable plaques and related therapeutic implications," *Nature Clinical Practice Cardiovascular Medicine*, vol. 2, pp. 456–464, 2005.

[20] J. Jansson, T. Nilsson, and O. Johnson, "Von Willebrand Factor in plasma: a novel risk for recurrent myocardial infarction and death," *British Heart Journal*, vol. 66, pp. 351–355, 1991.

[21] G. Stoll, C. Kleinschnitz, and B. Nieswandt, "Molecular mechanism of thrombus formation in ischemic stroke: novel insights and targets for treatment," *Blood*, vol. 112, pp. 3555–3562, 2008.

[22] S. De Castro, D. Cartoni, M. Fiorelli, M. Rasura, A. Anzini, E. Zanette, M. Beccia, C. Colonnese, F. Fedele, C. Fieschi, and N. Pandian, "Morphological and functional characteristics of patent foramen ovale and their embolic implications," *Stroke*, vol. 10, pp. 2407–13, 2000.

[23] R. Turner and R. Vink, "Inhibition of neurogenic inflammation as a novel treatment for ischemic stroke," *Cardiovascular Medicine*, vol. 11, p. E24, 2007.

[24] D. Escudero Augusto, L. Marqués Alvarez, and F. Taboada Costa, "Update in spontaneous cerebral hemorrhage," *Medicina Intensiva*, vol. 32, pp. 282–295, 2008.

[25] K. Machlus, J. Thon, and J. Italiano, "Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation," *British Journal of Haematology*, vol. 165, pp. 227–36, 2014.

[26] J. George, "Platelet immunoglobulin G: its significance for the evaluation of thrombocytopenia and for understanding the origin of alpha-granule proteins," *Blood*, vol. 76, p. 859, 1990.

[27] J. Lopez, R. Andrews, V. Afshar-Kharghan, and M. Berndt, "Bernard-Soulier syndrome," *Blood*, vol. 91, pp. 4397–418, 1998.

[28] S. Santoro and M. Zutter, "The alpha2beta1 integrin: a collagen receptor on platelets and other cells," *Thrombosis and Haemostasis*, vol. 74, pp. 813–21, 1995.

[29] A. Lusis, "Atherosclerosis," *Nature*, vol. 407, pp. 233–241, 2000.

[30] AHA, "Heart disease and stroke statistics - 2003 Update," *Dallas, American Heart Association*, 2002.

[31] L. Urbano and J. Bogousslavsky, "Antiplatelet Drugs in Ischemic Stroke Prevention: From Monotherapy to Combined Treatment," *Journal of Stroke and Cerebrovascular Diseases*, vol. 17, pp. 74–80, 2004.

[32] C. Patrono, "Aspirin as an antiplatelet drug," *New England Journal of Medicine*, vol. 330, pp. 1287–1294, 1994.

[33] T. Brott, H. Adams, C. Olinger, J. Marler, W. Barsan, J. Biller, J. Spilker, R. Holleran, R. Eberle, Hertzberg *et al.*, "Measurement of Acute Cerebral Infarction: A Clinical Examination Scale," *Stroke*, vol. 20, p. 7, 1989.

[34] J. Grotta, D. Chiu, M. Lu, S. Patel, S. Levine, B. Tilley, T. Brott, E. Haley, P. Lyden, R. Kothari, M. Frankel, C. Lewandowski, R. Libman, T. Kwiatkowski, J. Broderick, J. Marler, J. Corrigan, S. Huff, P. Mitsias, S. Talati, and D. Tanne, "Agreement and Variability in the Interpretation of Early CT Changes in Stroke Patients Qualifying for Intravenous rtPA Therapy," *Stroke*, vol. 30, pp. 1528–1533, 1999.

[35] S. Warach, J. Gaa, B. Siewert, P. Wielopolski, and R. Edelman, "Acute human stroke studied by whole brain echo planar diffusion-weighted magnetic resonance imaging," *Annals of Neurology*, vol. 37, pp. 231–241, 1994.

[36] M. Fisher and J. Saver, "Future directions of acute ischaemic stroke therapy," *Lancet Neurology*, vol. 14(7), no. 758-67, 2015.

[37] M. Lansberg, M. Straka, S. Kemp, M. Mlynash, L. Wechsler, T. Jovin, M. Wilder, H. Lutsep, T. Czartoski, R. Bernstein, C. Chang, S. Warach, F. Fazekas, M. Inoue, A. Tipirneni, S. Hamilton, G. Zaharchuk, M. Marks, R. Bammer, and G. Albers, "MRI profile and response to endovascular reperfusion after stroke (DEFUSE 2): a prospective cohort study," *Lancet Neurology*, vol. 11(10), no. 860-7, 2012.

[38] U. Dirnagl, C. Iadecola, and M. Moskowitz, "Pathobiology of ischaemic stroke: an integrated view," *Trends in Neuroscience*, vol. 22, pp. 391–7, 1999.

[39] "The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group: Tissue plasminogen activator for acute ischemic stroke," *New England Journal of Medicine*, vol. 333, pp. 1581–7, 1995.

[40] W. Hacke, M. Kaste, E. Bluhmki, M. Brozman, A. Dávalos, D. Giudetti, V. Larrue, K. Lees, Z. Medeghri, T. Machnig, D. Schneider, R. von Kummer, N. Wahlgren, and D. Toni, "Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke," *New England Journal of Medicine*, vol. 359, p. 13, 2008.

[41] H. Ma, B. Campbell, M. Parsons *et al.*, "Thrombolysis Guided by Perfusion Imaging up to 9 Hours after Onset of Stroke," *New England Journal of Medicine*, vol. 380(19), pp. 1795–1803, 2019.

[42] G. Thomalla, C. Simonsen, F. Boutitie *et al.*, "MRI-Guided Thrombolysis for Stroke with Unknown Time of Onset," *New England Journal of Medicine*, vol. 379, pp. 611–622, 2018.

[43] M. Goyal, B. Menon, W. van Zwam *et al.*, "Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomized trials," *Lancet*, vol. 387, pp. 1723–31, 2016.

[44] R. Nogueira, A. Jadhav, D. Haussen *et al.*, "Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct," *New England Journal of Medicine*, vol. 378, pp. 11–21, 2018.

[45] "WHO MONICA Project Principal Investigators: he World Health Organization MONICA Project (monitoring trends and determinants in cardiovascular disease): a major international collaboration," *Journal of Clinical Epidemiology*, vol. 41(2), pp. 105–114, 1988.

[46] G. Albers, L. Caplan, J. Easton, P. Fayad, J. Mohr, J. Saver, and D. Sherman, "Transient Ischemic Attack – Proposal for a New Definition," *New England Journal of Medicine*, vol. 347, pp. 1713–1716, 2002.

[47] J. Easton, J. Saver, G. Albers *et al.*, "Definition and evaluation of transient ischemic attack: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 40, pp. 2276–93, 2009.

[48] B. Hotter, I. Galinovic, C. Kunze, P. Brunecker, G. Jungehulsing, A. Villringer, M. Endres, K. Villringer, and J. Fiebach, "High-resolution diffusion-weighted imaging identifies ischemic lesions in a majority of transient ischemic attack patients," *Annals of Neurology*, vol. 86, pp. 452–457, 2019.

[49] S. Johnston, D. Gress, W. Browner, and S. Sidney, "Short-term prognosis after emergency department diagnosis of TIA," *JAMA*, vol. 284, pp. 2901–6, 2000.

[50] S. Coutts, J. Modi, and S. Patel, "What causes disability after transient ischemic attack and minor stroke? Results from the CT and MRI in the triage of TIA and minor Cerebrovascular events to Identify High Risk Patients," *Stroke*, vol. 43, pp. 3018–22, 2012.

[51] A. Yu, M. Hill, and S. Coutts, "Should minor stroke patients be thrombolyzed? A focused review and future directions," *International Journal of Stroke*, vol. 10(3), pp. 292–7, 2015.

[52] J. Alpert, K. Thygesen, E. Antman, and J. Bassand, "Myocardial infarction redefined - a consensus document of the joint European society of Cardiology/American college of cardiology committee for the redefinition of myocardial infarction," *Journal American College Cardiology*, vol. 36(3), pp. 959–969, 2000.

[53] V. Tapson, "Acute pulmonary embolism," *New England Journal of Medicine*, vol. 358(10), pp. 1037–1052, 2008.

[54] W. Whiteley, M. Tseng, and P. Sandercock, "Blood biomarkers in the diagnosis of ischemic stroke: a systematic review," *Stroke*, vol. 39(10), pp. 2902–9, 2008.

[55] M. Maas and K. Furie, "Molecular biomarkers in stroke diagnosis and prognosis," *Biomarkers in Medicine*, vol. 3(4), pp. 363–383, 2009.

[56] D. Kögel, M. Peters, H. König, S. Hashemi, N. Bui, V. Arolt, M. Rothermund, and J. Prehn, "S100B potently activates p65/c-Rel transcriptional complexes in hippocampal neurons: Clinical implications for the role of S100B in excitotoxic brain injury," *Neuroscience*, vol. 127(4), pp. 913–20, 2004.

[57] M. Rothermund, M. Peters, J. Prehn, and V. Arolt, "S100B in brain damage and neurodegeneration," *Microscopic Research and Technique*, vol. 60(6), pp. 614–632, 2003.

[58] C. Foerch, M. Wunderlich, F. Dvorak, M. Humpich, T. Kahles, M. Goertler, J. Alvarez-Sabin, C. Wallesch, C. Molina, H. Steinmetz, M. Sitzer, and J. Montaner, "Elevated serum S100B levels indicate a higher risk of hemorrhagic transformation after thrombolytic therapy in acute ischemic stroke," *Stroke*, vol. 38(9), pp. 2491–5, 2007.

[59] L. Pelinka, A. Kroepfl, R. Schmidhammer, M. Krenn, W. Buchinger, H. Redl, and A. Raabe, "Glial fibrillary acidic protein in serum after traumatic brain injury and multiple trauma," *Journal of Trauma*, vol. 57(5), pp. 1006–12, 2004.

[60] M. Hill, G. Jackowski, N. Bayer, M. Lawrence, and R. Jaeschke, "Biochemical markers in acute ischemic stroke," *CMAJ*, vol. 162(8), pp. 1139–1140, 2000.

[61] M. Herrmann, P. Vos, M. Wunderlich, C. de Bruijn, and K. Lamers, "Release of glial tissue-specific proteins after acute stroke: a comparative analysis of

serum concentrations of protein S-100B and glial fibrillary acidic protein," *Stroke*, vol. 31, pp. 2670–2677, 2000.

[62] E. Jauch, C. Lindsell, J. Broderick, S. Fagan, B. Tilley, and S. Levine, "Association of serial biochemical markers with acute ischemic stroke: The National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator Stroke Study," *Stroke*, vol. 37(10), pp. 2508–13, 2006.

[63] M. Casmiro, S. Maitan, F. De Pasquale, V. Cova, E. Scarpa, and L. Vignatelli, "Cerebrospinal fluid and serum neuron-specific enolase concentrations in a normal population," *European Journal of Neurology*, vol. 12(5), pp. 369–74, 2005.

[64] S. Tiedt, M. Duering, C. Barro, A. Gizem Kaya, J. Boeck, F. Bode, M. Klein, F. Dorn, B. Gieserich, L. Kellert, B. Ertl-Wagner, M. Goertler, G. Petzold, J. Kuhle, F. Wollenweber, N. Peters, and M. Dichgans, "Serum neurofilament light: A biomarker of neuroaxonal injury after ischemic stroke," *Neurology*, vol. 91, pp. e1338–1347, 2018.

[65] S. Tiedt, M. Prestel, R. Malik, N. Schieferdecker, M. Duering, V. Kautzky, I. Stoycheva, J. Boeck, B. Northoff, M. Klein, F. Dorn, K. Krohn, D. Teupser, A. Liesz, N. Plesnila, L. Holdt, and M. Dichgans, "RNA-Seq Identifies Circulating miR-125a-5p, miR-125b-5p and miR-143-3p as Potential Biomarkers for Acute Ischemic Stroke," *Circulation Research*, vol. 121, pp. 970–980, 2017.

[66] N. Tran, V. Wong, S. Schreiber, J. Bready, and M. Fisher, "Regulation of brain capillary endothelial thrombomodulin mRNA expression," *Stroke*, vol. 27(12), pp. 2301–2304, 1996.

[67] J. Olivot, J. Labreuche, T. de Broucker, O. Poirier, F. Cambien, M. Aiach, and P. Amarenco, "Thombomodulin gene polymorphisms in brain infarction and mortality after stroke," *Journal of Neurology*, vol. 225(4), pp. 514–9, 2008.

[68] E. Nomura, T. Kohriyama, K. Kozuka, H. Kajikawa, S. Nakamura, and M. Matsumoto, "Significance of serum soluble thrombomodulin level in acute cerebral infarction," *European Journal of Neurology*, vol. 11(5), pp. 329–334, 2004.

[69] N. Beamer, B. Coull, W. Clark, J. Hazel, and J. Silberger, "Interleukin-6 and Interleukin-1 receptor antagonist in acute stroke," *Annals of Neurology*, vol. 37(6), pp. 800–805, 1995.

[70] M. Di Napoli, F. Papa, and V. Bocola, "Prognostic influence of increased C-reactive protein and fibrinogen levels in ischemic stroke," *Stroke*, vol. 32(1), pp. 133–138, 2001.

[71] W. Feinberg, L. Erickson, D. Bruck, and J. Kittelson, "Hemostatic markers in acute ischemic stroke. Association with stroke type, severity, and outcome," *Stroke*, vol. 27(8), pp. 1296–1300, 1996.

[72] A. Carter, A. Catto, M. Mansfield, J. Bamford, and P. Grant, "Predictive variables for mortality after acute ischemic stroke," *Stroke*, vol. 38(6), pp. 1873–1880, 2007.

[73] T. Tombul, C. Atbas, and O. Anlar, "Hemostatic markers and platelet aggregation factors as predictive markers for type of stroke and neurological disability following cerebral infarction," *Journal of Clinical Neuroscience*, vol. 12(4), pp. 429–34, 2005.

[74] E. Haapaniemi and T. Tatlisumak, "Is D-dimer helpful in evaluating stroke patients? A systematic review," *Acta Neurologica Scandinavica*, vol. 119(3), pp. 141–50, 2009.

[75] J. Oppenheim, "Cytokines: past, present, and future," *International Journal of Hematology*, vol. 74(1), pp. 3–8, 2001.

[76] H. Emsley, C. Smith, C. Gavin, R. Georgiou, A. Vail, E. Barberan, K. Illingworth, S. Scarth, V. Wickramasinghe, M. Hoadley, N. Rothwell, P. Tyrrell, and S. Hopkins, "Clinical outcome following acute ischemic stroke relates to both activation and auto regulatory inhibition of cytokine production," *BMC Neurology*, vol. 28, p. 7:5, 2007.

[77] J. Casas, T. Shah, A. Hingorani, J. Danesh, and M. Pepys, "C-reactive protein and coronary heart disease: a critical review," *Journal of Internal Medicine*, vol. 264(4), pp. 295–314, 2008.

[78] N. Rost, P. Wolf, C. Kase, M. Kelly-Hayes, H. Silbershatz, J. Massaro, R. D'Agostino, C. Franzblau, and P. Wilson, "Plasma concentration of C-reactive protein and risk of ischemic stroke transient ischemic attack: The Framingham Study," *Stroke*, vol. 32(11), pp. 2575–9, 20021.

[79] C. Smith, H. Emsley, C. Gavin, R. Georgiou, A. Vail, E. Barberan, G. del Zoppo, J. Hallenbeck, N. Rothwell, S. Hopkins, and P. Tyrrell, "Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome," *BMC Neurology*, vol. 15, p. 4:2, 2004.

[80] J. Arenillas, J. Alvarez-Sabin, C. Molina, P. Chacón, J. Montaner, A. Rovira, B. Ibarra, and M. Quintana, "C-reactive protein predicts further ischemic events in first-ever transient ischemic attack or stroke patients with intracranial large-artery occlusive disease," *Stroke*, vol. 34(10), pp. 2463–8, 2003.

[81] N. Anand and L. Stead, "Neuron-specific enolase as a marker for acute ischemic stroke: a systematic review," *Cerebrovascular Disease*, vol. 20(4), pp. 213–219, 2005.

[82] L. Allard, N. Turck, P. Burkhard, N. Walter, A. Rosell, M. Gex-Fabry, D. Hochstrasser, J. Montaner, and J. Sanchez, "Ubiquitin fusion degradation protein 1 as a blood marker for the early diagnosis of ischemic stroke," *Biomarker Insights*, vol. 19;2, pp. 155–64, 2007.

[83] M. Vogelbaum, T. Masaryk, P. Mazzone, T. Mekhail, V. Fazio, S. McCartney, N. Marchi, A. Kanner, and D. Janigro, "S100B as a predictor of brain metastases: brain versus cerebrovascular damage," *Cancer*, vol. 104(4), pp. 817–24, 2005.

[84] D. Bartel, "microRNAs: genomics, biogenesis, mechanism, and function," *Cell*, no. 116, pp. 281–297, 2004.

[85] R. Lee, R. Feinbaum, and V. Ambros, "The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," *Cell*, vol. 75(5), pp. 843–54, 1993.

[86] S. Griffiths-Jones, H. Saini, S. van Dongen, and A. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acid Research*, vol. 36, pp. D154–8, 2008.

[87] J. Krol, I. Loedige, and W. Filipowicz, "The widespread regulation of microRNA biogenesis, function and decay," *Nature Review Genetics*, vol. 11(9), pp. 597–610, 2010.

[88] E. van Rooij and E. Olson, "MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles," *Nature Reviews Drug Discovery*, vol. 11, pp. 860–872, 2012.

[89] A. Schober, M. Nazari-Jahantigh, Y. Wei, K. Bidzhekov, F. Gremse, J. Grommes, R. Megens, K. Heyll, H. Noels, M. Hristov, S. Wang, F. Kiessling, E. Olson, and C. Weber, "MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1," *Nature Medicine*, vol. 20, pp. 368–76, 2014.

[90] M. Klein, S. Impey, and R. Goodman, "Role reversal: the regulation of neuronal gene expression by microRNAs," *Current Opinion in Neurobiology*, vol. 15(5), pp. 507–513, 2005.

[91] A. Giraldez, R. Cinalli, M. Glasner, A. Enright, J. Thomson, S. Baskerville, S. Hammond, D. Bartel, and A. Schier, "MicroRNAs regulate brain morphogenesis in zebrafish," *Science*, vol. 308, pp. 833–8, 2005.

[92] S. Hatfield, H. Shcherbata, K. Fischer, K. Nakahara, R. Carthew, and H. Ruohola-Baker, "Stem cell division is regulated by the microRNA pathway," *Nature*, vol. 435, pp. 974–978, 2005.

[93] C. Glinge, S. Clauss, K. Boddum, R. Jabbari, J. Jabbari, B. Risgaard, P. Tomsits, B. Hildebrand, S. Kääb, R. Wakili, T. Jespersen, and J. Tfelt-Hansen, "Stability of Circulating Blood-Based microRNAs – Pre-Analytic Methodological Considerations," *PLoS One*, vol. 12(2), p. 0167969, 2017.

[94] X. Chen, Y. Ba, L. Ma *et al.*, "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Research*, vol. 18, pp. 997–1006, 2008.

[95] V. Metzinger-Le Meuth, S. Burtey, P. Maitrias, Z. Massy, and L. Metzinger, "microRNAs in the pathophysiology of CKD-MBD: Biomarkers and innovative drugs," *Biochimica et biophysica acta. Molecular basis of disease*, vol. 1863(1), pp. 337–345, 2017.

[96] A. Zernecke, K. Bidzhekov, H. Noels, E. Shagdarsuren, L. Gan, B. Denecke, M. Hristov, T. Köppel, M. Jahantigh, E. Lutgens, S. Wang, E. Olson, A. Schober, and C. Weber, "Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection," *Science Signaling*, vol. 2, p. ra81, 2009.

[97] Y. Zhang, D. Liu, X. Chen, J. Li, L. Li, Z. Bian, F. Sun, J. Lu, Y. Yin, X. Cai, Q. Sun, K. Wang, Y. Ba, Q. Wang, D. Wang, J. Yang, P. Liu, T. Xu, Q. Yan, J. Zhang, K. Zen, and C. Zhang, "Secreted monocytic miR-150 enhances targeted endothelial cell migration," *Molecular Cell*, vol. 39, pp. 133–144, 2010.

[98] A. Etheridge, I. Lee, L. Hood, D. Galas, and K. Wang, "Extracellular microRNA: a new source of biomarkers," *Mutation Research*, vol. 717, pp. 85–90, 2011.

[99] E. Creemers, A. Tijsen, and Y. Pinto, "Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?" *Circulation Research*, vol. 110, pp. 483–95, 2012.

[100] G. Wang, J. Zhu, J. Zhang, Q. Li, Y. Li, J. He, Y. Qin, and Q. Jing, "Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans," *European Heart Journal*, vol. 31, pp. 659–666, 2010.

[101] M. Corsten, R. Dennert, S. Jochems, T. Kuznetsova, Y. Devaux, L. Hofstra, D. Wagner, J. Staessen, S. Heymans, and B. Schroen, "Circulating microRNA-208b and microRNA-499 reflect myocardial damage in cardiovascular disease," *Circulation. Cardiovascular Genetics*, vol. 3, pp. 499–506, 2010.

[102] S. Tiedt and M. Dichgans, "Role of Non-Coding RNAs in Stroke," *Stroke*, vol. 49, pp. 3098–3106, 2018.

[103] R. Flaumenhaft, J. Dilks, J. Richardson, E. Alden, S. Patel-Hett, E. Battinelli, G. Klement, M. Sola-Visner, and J. Italiano, "Megakaryocyte-derived microparticles: direct visualization and distinction from platelet-derived microparticles," *Blood*, vol. 113, pp. 1112–1121, 2009.

[104] J. Opalinska, A. Bersenev, Z. Zhang, A. Schmaier, J. Choi, Y. Yao, J. D'Souza, W. Tong, and M. Weiss, "MicroRNA expression in maturing murine megacaryocytes," *Blood*, vol. 116, pp. e128–138, 2010.

[105] M. Mayr, A. Zampetaki, P. Willeit, J. Willeit, and S. Kiechl, "MicroRNAs within the continuum of postgenomics biomarker discovery," *Arteriosclerosis, thrombosis, and vascular biology*, vol. 33, pp. 206–214, 2013.

[106] P. Willeit, A. Zampetaki, K. Dudek, D. Kaudewitz, A. King, N. Kirkby, R. Crosby-Nwaobi, M. Prokopi, I. Drozdov, S. Langley, S. Sivaprasad, H. Markus, J. Mitchell, T. Warner, S. Kiechl, and M. Mayr, "Circulating microRNAs as novel biomarkers for platelet activation," *Circulation Research*, vol. 112, pp. 595–600, 2013.

[107] A. Zampetaki, S. Kiechl, I. Drozdov, P. Willeit, U. Mayr, M. Prokopi, A. Mayr, S. Weger, F. Oberhollenzer, E. Bonora, A. Shah, J. Willeit, and M. Mayr,

"Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes," *Circulation Research*, vol. 107, pp. 810–817, 2010.

[108] N. Sunderland, P. Skroblin, T. Barwari, R. Huntley, R. Lu, A. Joshi, R. Lovering, and M. Mayr, "MicroRNA Biomarkers and Platelet Reactivity: The Clot Thickens," *Circulation Research*, vol. 120, pp. 418–435, 2017.

[109] C. Stratz, T. Nührenberg, H. Binder, C. Valina, D. Trenk, W. Hochholzer, F. Neumann, and B. Fiebich, "Microarray profiling exhibits remarkable intra-individual stability of human platelet microRNAs," *Thrombosis and Haemostasis*, vol. 107, pp. 634–641, 2012.

[110] O. Gidlöf, M. van der Brug, J. Ohman, P. Gilje, B. Olde, C. Wahlestedt, and D. Erlinge, "Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression," *Blood*, vol. 121, pp. 3908–3917, 2013.

[111] A. Elgheznawy, L. Shi, J. Hu, I. Wittig, H. Laban, J. Pircher, A. Mann, P. Provost, V. Randriamboavonjy, and I. Fleming, "Dicer cleavage by calpain determines platelet microRNA levels and function in diabetes," *Circulation Research*, vol. 117, pp. 157–165, 2015.

[112] H. Liang, X. Yan, Y. Pan, Y. Wang, N. Wang, L. Li, Y. Liu, X. Chen, C. Zhang, H. Gu, and K. Zen, "MicroRNA-223 delivered by platelet-derived microvesicles promotes lung cell cancer invasion via targeting tumor suppressor EPB41L3," *Molecular Cancer*, vol. 14, p. 58, 2015.

[113] D. Kaudewitz, P. Skroblin, L. Bender, T. Barwari, P. Willeit, R. Pechlaner, N. Sunderland, K. Willeit, A. Morton, P. Armstrong, M. Chan, R. Lu, X. Yin, F. Gracio, K. Dudek, S. Langley, A. Zampetaki, E. de Rinaldis, S. Ye, T. Warner, A. Saxena, S. Kiechl, R. Storey, and M. Mayr, "Association of microRNAs and YRNAs with platelet function," *Circulation Research*, vol. 118, pp. 420–432, 2016.

[114] G. Llovera, S. Roth, N. Plesnila, R. Veltkamp, and A. Liesz, "Modeling Stroke in Mice: Permanent Coagulation of the Distal Middle Cerebral Artery," *Journal of Visualized Experiments*, vol. 89, p. e51729, 2014.

[115] M. McAlexander, M. Phillips, and K. Witwer, "Comparison of Methods for miRNA Extraction from Plasma and Quantitative Recovery of RNA from Cerebrospinal Fluid," *Frontiers in Genetics*, vol. 4, p. 83, 2013.

[116] D. Kaudewitz, R. Lee, P. Willeit, R. McGregor, H. Markus, S. Kiechl, A. Zampetaki, R. Storey, K. Channon, and M. Mayr, "Impact of intravenous heparin on quantification of circulating microRNAs in patients with coronary artery disease," *Thrombosis and Haemostasis*, vol. 119, pp. 609–615, 2013.

[117] J. Boeckel, C. Thome, D. Leistner, A. Zeiher, S. Fichtlscherer, and S. Dimmeler, "Heparin selectively affects quantification of microRNAs in human blood samples," *Clinical Chemistry*, vol. 59, pp. 1125–1127, 2013.

[118] A. Chaudhuri, S. Yelamanchili, and H. Fox, "Combined fluorescent in situ hybridization for detection of microRNAs and immunofluorescent labeling for cell-type markers," *Frontiers in Cellular Neuroscience*, vol. 7, p. 160, 2013.

[119] L. Edelstein, S. McKenzie, C. Shaw, M. Holinstat, S. Kunapuli, and P. Bray, "MicroRNAs in platelet production and activation," *Thrombosis and Haemostasis*, vol. 11, pp. 340–50, 2013.

[120] J. Lu, S. Guo, B. Ebert, H. Zhang, X. Peng, J. Bosco, J. Pretz, R. Schlanger, J. Wang, R. Mak, D. Dombkowski, F. Preffer, D. Scadden, and T. Golub, "MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors," *Developmental Cell*, vol. 14, pp. 843–53, 2008.

[121] R. Georgantas, R. Hildreth, S. Morisot, J. Alder, C. Liu, S. Heimfeld, G. Calin, C. Croce, and C. Civin, "CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control," *Proceedings of the National Academy of Sciences of the USA*, vol. 104, pp. 2750–5, 2007.

[122] A. Zampetaki, P. Willeit, L. Tilling, I. Drozdov, M. Prokopi, J. Renard, A. Mayr, S. Weger, G. Schett, A. Shah, C. Boulanger, J. Willeit, P. Chowienczyk, S. Kiechl, and M. Mayr, "Prospective study on circulating microRNAs and risk of myocardial infarction," *Journal American College Cardiology*, vol. 60, pp. 290–299, 2012.

[123] P. Landry, I. Plante, D. Ouellet, M. Perron, G. Rousseau, and P. Provost, "Existence of a microRNA pathway in anucleate platelets," *Nature structural and molecular biology*, vol. 16, pp. 961–966, 2009.

[124] R. Shi, L. Ge, Y. Zhou, W. Ji, R. Lu, Y. Zhang, S. Zeng, X. Liu, J. Zhao, W. Zhang, T. Jiang, and Y. Li, "Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity," *Thrombosis Research*, vol. 131, pp. 508–513, 2013.

[125] M. Lagos-Quintana, R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel, and T. Tuschl, "Identification of Tissue-specific microRNAs from Mouse," *Current Biology*, vol. 12, pp. 735–739, 2002.

[126] M. McCall, H. McMurray, H. Land, and A. Almudevar, "On non-detects in qPCR data," *Bioinformatics*, vol. 30(16), pp. 2310–2316, 2014.

[127] R. Mayeux, "Biomarkers: Potential Uses and Limitations," *NeuroRX*, vol. 1(2), pp. 182–188, 2004.

[128] J. Hopyan, A. Ciarallo, D. Dowlatshahi, P. Howard, V. John, R. Yeung, L. Zhang, J. Kim, G. MacFarlane, T. Lee, and R. Aviv, "Certainty of stroke diagnosis: incremental benefit with CT perfusion over noncontrast CT and CT-angiography," *Radiology*, vol. 255, pp. 142–153, 2010.

[129] Y. D'Allessandra, P. Devanna, F. Limana *et al.*, "Circulating microRNAs are new and sensitive biomarkers of myocardial infarction," *European Heart Journal*, vol. 31(22), pp. 2765–73, 2010.

[130] C. Liebetrau, H. Möllmann, O. Dörr, S. Szardien, C. Troidl, M. Willmer, S. Voss, L. Gaede, J. Rixe, A. Rolf, C. Hamm, and H. Nef, "Release kinetics of circulating muscle-enriched microRNAs in patients undergoing transcoronary ablation of septal hypertrophy," *Journal of the American College Cardiology*, vol. 62, pp. 992–998, 2013.

[131] E. Jauch, J. Saver, H. Adams, A. Bruno, J. Connors, B. Demaerschalk, P. Khatri, P. McMullan, A. Qureshi, K. Rosenfield, P. Scott, D. Summers, D. Wang, M. Wintermark, and H. Yonas, "Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 44, pp. 870–947, 2013.

[132] D. Guo, J. Liu, W. Wang, F. Hao, X. Sun, X. Wu, P. Bu, Y. Zhang, Y. Liu, F. Liu, Q. Zhang, and F. Jiang, "Alteration in abundance and compartmentalization of inflammation-related miRNAs in plasma after intracerebral hemorrhage," *Stroke*, vol. 44(6), pp. 1739–42, 2013.

[133] S. Dolmans, F. Rutten, N. Koenen, M. Bartelink, J. Reitsma, L. Kappelle, and A. Hoes, "Candidate Biomarkers for the Diagnosis of Transient Ischemic Attack: a Systematic Review," *Cerebrovascular Disease*, vol. 47, pp. 207–216, 2019.

[134] J. McDonald, D. Milosevic, H. Reddi, S. Grebe, and A. Algeciras-Schimnich, "Analysis of circulating microRNA: preanalytical and analytical challenges," *Clinical Chemistry*, vol. 57, pp. 833–840, 2011.

[135] H. De Boer, C. van Solingen, J. Prins, J. Duijs, M. Huisman, T. Rabelink, and A. van Zonnewald, "Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease," *European Heart Journal*, vol. 44, pp. 3451–3457, 2013.

[136] K. Jeyaseelan, K. Lim, and A. Armugam, "MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion," *Stroke*, vol. 39, pp. 959–966, 2008.

[137] A. Dharap, K. Bowen, R. Place, L. Li, and R. Vemuganti, "Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome," *Journal of Cerebral Blood Flow and Metabolism*, vol. 29, pp. 675–687, 2009.

[138] C. Sommer, "Ischemic stroke: experimental models and reality," *Acta Neuropathologica*, vol. 133(2), pp. 245–261, 2017.

[139] J. Xiao, B. Shen, J. Li, D. Lv, Y. Zhao, F. Wang, and J. Xu, "Serum microRNA-499 and microRNA-208a as biomarkers of acute myocardial infarction," *International Journal of Clinical and Experimental Medicine*, vol. 7(1), pp. 136–141, 2014.

[140] B. Buller, X. Liu, X. Wang, R. Zhang, L. Zhang, A. Hozeska-Solgot, M. Chopp, and Z. Zhang, "MicroRNA-21 protects neurons from ischemic death," *FEBS Journal*, vol. 227, pp. 4299–307, 2010.

[141] W. Kim, Y. Lee, N. McKenna, M. Yi, F. Simunovic, Y. Wang, B. Kong, R. Rooney, H. Seo, R. Stephens, and K. Sonntag, "miR-126 contributes to Parkinson's disease by dysregulating the insulin-like growth factor/phosphoinositide 3-kinase signaling," *Neurobiology Aging*, vol. 35(7), pp. 1712–1721, 2014.

[142] M. Ziats and O. Rennert, "Identification of differentially expressed microRNAs across the developing human brain," *Molecular Psychiatry*, vol. 19(7), pp. 848–52, 2014.

[143] B. Morquette, C. Juzwik, S. Drake *et al.*, "MicroRNA-223 protects neurons from degeneration in experimental autoimmunencephalomyelitis," *Brain*, vol. 142, pp. 2979–2995, 2019.

[144] J. Miguel-Hidalgo, K. Hall, H. Bonner, A. Roller, M. Syed, C. Park, J. Ball, M. Rothenberg, C. Stockmeier, and D. Romero, "MicroRNA-21: Expression in oligodendrocytes and correlation with low myelin mRNAs in depression and alcoholism," *Progress in neuro-psychopharmacology and biological psychiatry*, vol. 79, pp. 503–514, 2017.

[145] C. Sabatel, L. Malvaux, N. Bovy, C. Deroanne, V. Lambert, M. Gonzalez, A. Colige, J. Rakic, A. Noel, J. Martial, and I. Struman, "MicroRNA-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells," *PLoS One*, vol. 6(2), p. e16979, 2011.

[146] P. Tsai, Y. Liao, Y. Wang, H. Lin, R. Lin, and S. Juo, "Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease," *Journal of Vascular Research*, vol. 50, pp. 346–54, 2013.

[147] J. Zhou and J. Zhang, "Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction," *Molecular Medicine Reports*, vol. 10, pp. 971–6, 2014.

[148] M. Meloni, M. Marchetti, K. Garner, B. Littlejohns, G. Sala-Newby, N. Xenophontos, I. Floris, M. Suleiman, P. Madeddu, A. Caporali, and C. Emanueli, "Local inhibition of microRNA-24 improves reparative angiogenesis and left ventricle remodeling and function in mice with myocardial infarction," *Molecular Therapy*, vol. 21(7), pp. 1390–402, 2013.

[149] S. Wang, A. Aurora, B. Johnson, X. Qi, J. McAnally, J. Hill, J. Richardson, R. Bassel-Duby, and E. Olson, "The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis," *Developmental Cell*, vol. 15, pp. 261–271, 2008.

[150] T. Harris, M. Yamakuchi, M. Ferlito, J. Mendell, and C. Lowenstein, "microRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1," *Proceedings of the National Academy of Sciences of the USA*, vol. 105, pp. 1516–21, 2008.

[151] J. Gao, S. Yang, K. Wang, Q. Zhong, A. Ma, and X. Pan, "Plasma miR-126 and miR-143 as Potential Novel Biomarkers for Cerebral Atherosclerosis," *Journal of Cerebral and Vascular Diseases*, vol. 1, pp. 38–43, 2019.

[152] F. Jin and J. Xing, "Circulating miR-126 and miR-130a levels correlate with lower disease risk, disease severity and reduced inflammatory cytokine levels in acute ischemic stroke patients," *Journal of Neurological Sciences*, vol. 39, pp. 1757–1765, 2018.

[153] G. Hollopeter, H. Jantzen, D. Vincent, G. Li, L. England, V. Ramakrishnan, R. Yang, P. Nurden, A. Nurden, D. Julius, and P. Conley, “Identification of the platelet ADP receptor targeted by antithrombotic drugs,” *Nature*, vol. 409, pp. 202–7, 2001.

[154] Y. Chen, Y. Song, J. Huang, M. Qu, Y. Zhang, J. Geng, Z. Zhang, J. Liu, and G. Yang, “Increased Circulating Exosomal miRNA-223 is associated with acute ischemic stroke,” *Frontiers in Neurology*, vol. 8, p. 57, 2017.

[155] P. Mestdagh, P. van Vlierberghe, A. de Weer, D. Muth, F. Westermann, F. Spelemann, and J. Vandesompele, “A novel and universal method for microRNA RT-qPCR data normalization,” *Genome Biology*, vol. 10, p. R64, 2009.

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