Chapter 9
microRNAs in Cerebrovascular Disease

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Abstract Cardiovascular diseases are major causes of morbidity and mortality in developed countries. Cerebrovascular diseases, especially stroke, represent major burden of disability and economy impact. Major advances in primary and secondary prevention and therapy are needed in order to tackle this public health problem. Our better understanding of pathophysiology is essential in order to develop novel diagnostic and therapeutic tools and strategies. microRNAs are a family of important post-transcriptional regulators of gene expression and their involvement in the pathophysiology of cerebrovascular diseases has already been reported. Moreover, microRNAs may represent above-mentioned potential diagnostic and therapeutic tools in clinical practice. Within this chapter, we briefly describe basic epidemiology, aetiology and clinical manifestation of following cerebrovascular diseases: extracranial carotid atherosclerosis, acute stroke, intracranial aneurysms and cerebral arterio-venous malformations. Further, in each chapter, the current knowledge about

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the involvement of specific microRNAs and their potential use in clinical practice will be summarized. More specifically, within the subchapter "miRNAs in carotid atherosclerosis", general information about miRNA involvement in atherosclerosis will be described (miR-126, miR-17-92, miR-155 and others) with special emphasis put on miRNAs affecting carotid plaque progression and stability (e.g. miR-145, miR-146 or miR-217). In the subchapter "miRNAs in acute stroke", we will provide insight into recent knowledge from animal and human studies concerning miRNA profiling in acute stroke and their expression dynamics in brain tissue and extracellular fluids (roles of, e.g. let-7 family, miR-21, miR-29 family, miR-124, miR-145, miR-181 family, miR-210 and miR-223). Subchapters dealing with "miRNAs and AV malformations" and "miRNAs and intracranial aneurysms" will focus on miR-21, miR-26, miR-29 family and miR-143/145.

Keywords microRNA • Carotid atherosclerosis • Ischemic stroke • Intracranial aneurysms • Cerebral arterio-venous malformations

Carotid Atherosclerosis

Epidemiology of Carotid Atherosclerosis

Atherosclerosis represents a complex arterial disease characterized by vascular wall inflammation and remodelling in the end resulting in the creation of atherosclerotic plaques [1]. According to the site of plaques occurrence, atherosclerosis clinically presents as coronary artery disease (CAD), carotid atherosclerosis (Fig. 9.1) or peripheral artery disease (PAD) [2]. Carotid arteries represent one of the specific places of plaques accumulation, especially concerning the ostium and bifurcation of common carotid artery, probably due to alteration of blood flow at these locations [3]. Accumulation of atherosclerotic plaques in carotid arteries

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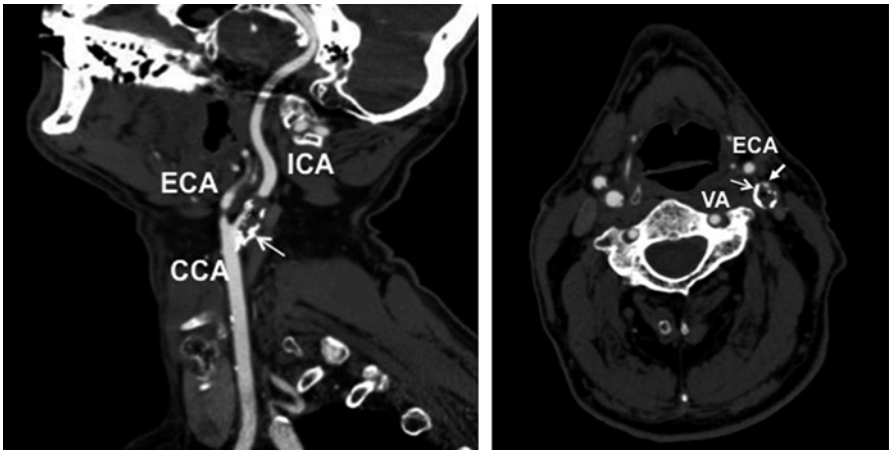

Fig. 9.1 Atherosclerotic plaque in the left internal carotid artery, curved multiplanar reconstruction (MPR). Sagittal (*left*) and axial (*right*) CT angiography scans of the neck demonstrating >90 % narrowing of the internal carotid artery behind the common carotid artery bifurcation. Atherosclerotic plaque is represented by a hypodense area in the arterial lumen (*black star*), *open arrows* point to the plaque calcifications (the most apparent hyperdense part; on the axial scan forming a hyperdense rim). *White arrow* on the axial scan (*right*) shows the residual vascular lumen (diminished by mass of the plaque). *CCA* common carotid artery, *ECA* external carotid artery, *ICA* internal carotid artery, *VA* vertebral artery

affects significantly blood supply to the brain and predisposes patients to fainting, syncope and possibly to ischemic stroke. Based on data of Framingham cohort study, the reported prevalence of carotid artery atherosclerosis (defined as stenosis >50 % evaluated by carotid ultrasound) in the study population was 7 % in women and 9 % in men [4–6]. In term of stroke aetiology, the atherosclerotic aetiology accounts for 10–15 % of ischemic strokes and soon revelation of carotid atherosclerosis represents a risk factor for future stroke development [7].

Clinical Evaluation of Carotid Atherosclerosis

Carotid stenosis can be evaluated by various non-invasive methods [8, 9]:

- *B-mode ultrasound* (parameters as intima-media thickness (IMT), echogenic quality and 3D volume measurement of plaque characteristics)
- *Transcranial Doppler* (microemboli detection used as a marker of plaque instability; detection of intracranial carotid stenosis)
- *Computed tomography angiography/CTA* (assessment of arterial stenosis and basic plaque characteristics)
- *Magnetic resonance imaging/MRI* (evaluating the intraplaque haemorrhage and presence of a fibrous cap, both representing radiological markers of plaque vulnerability)

- *Positron emission tomography/PET* (research imaging technique used for evaluation of vascular wall inflammation)

Periodical carotid plaque evaluation represents a tool for therapy monitoring and carotid plaque evolution. The most important is a proper treatment of vascular risk factors (hypertension, diabetes, dyslipidemia, smoking cessation, etc.), specifically, the therapy of dyslipidemia is typically led by statins which lower serum cholesterol levels, reduce subclinical inflammation inside the plaque, and promote intraplaque stabilization independently of cholesterol lowering as well [10].

Pathophysiology of Atherosclerosis and Roles of miRNAs

From the pathophysiological point of view, atherosclerosis represents a complex process of arterial wall inflammation and remodelling that includes activation of endothelial cells (ECs) by various external stimuli, e.g. disrupted blood-flow [3] or oxidized LDL (oxLDL) presence [11], which further leads to activation/proliferation of vascular smooth muscle cells (VSMCs), vascular wall fibroblasts and also other cell types, such as platelets, adipocytes or macrophages (and their altered form "foam cells") [1, 11]. All above-mentioned processes results in the end in the creation of atherosclerotic plaques. Predominantly, atherosclerotic plaques are formed at places exposed to disrupted blood flow, in vivo specifically in the aortic arch and arterial bifurcations [12]. Recent advances in our understanding of atherosclerosis pathophysiology revealed that shear stress significantly affects the gene expression of EC exposed to either high or low shear stress, and miRNAs seem to participate even in the flow-regulation of atherosclerosis [12, 13].

Roles of miRNA in atherosclerosis include mainly (a) regulation of gene expression in all aforementioned cells and (b) miRNAs serve as mediators of cell-to-cell communication [13, 14]. From the atherosclerotic point of view, miRNAs (also termed "athero-miRs") can be divided to several groups concerning their individual characteristics—to mechano-responsive or mechano-irresponsive (depending on the response to shear-stress) or to proatherogenic, antiatherogenic or ambigual (according to their effects on atherosclerosis progression) [15]. Providing the complex overview of miRNAs involved in atherosclerosis is not the main goal in this subchapter—here we will provide just a brief summary of the roles of main atheromiRs, i.e. miR-17-92 cluster, miR-126, miR-143/145 cluster and miR-155, and then we will specifically focus on human studies dealing with carotid atherosclerosis.

AtheromiRs

Concerning the complex pathophysiology of atherosclerosis, from all potential miRNAs involved, we should definitely mention ,widespread miR-17-92 cluster [16], endothelium-specific miR-126 [17], vascular-smooth muscle, cell enriched

miR-143/145 cluster [18, 19], and also immunity-related miR-155, that is even tightly connected to macrophages function [20].

Members of miR-17-92 cluster (i.e. miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) were shown to be downregulated after the exposure of endothelial cells to laminar (≈stable) flow [21], which makes miR-17-92 proatherogenic—we can imagine that by downregulating miR-17-92, endothelium somehow "protects itself" from miR-17-92 effects. miR-17-92 was shown to target Krüppel-like factor (KLF)-2 and KLF-4 and this targeting promoted endothelial inflammation—on the other hand, if miR-17-92 was inhibited, it reduced the expression of proinflammatory markers [22]. Moreover, on the contrary to tumour angiogenesis that is promoted by miR-17-92 increase [23, 24], endothelial sprouting and neovascularization are promoted if miR-17-92 cluster activity is decreased [23, 24] and this may potentially contribute to restenosis occurrence. Concerning other general roles of miR-17-92 cluster in the regulation of cell cycle and apoptosis [25], more studies are needed to shed a light on the precise functions of this cluster in atherosclerotic process; however, inhibition of miR-17-92 cluster may generally be considered atheroprotective.

Endothelial specific miR-126 (and its passenger strand miR-126*) are both known to be highly expressed in endothelium and they are crucial for vascular integrity and the vessel development (by targeting PIK3R2, Spred-1 [17, 26], VEGF [27] or, e.g. by affecting angiopoietin signalling [28]). Their upregulation in endothelial cells generally seems to be atheroprotective. By targeting vascular cell adhesion molecule (VCAM)-1 miR-126 attenuates endothelial inflammation [29]. Similarly, miR-126 packaged in apoptotic bodies enhances endothelial recovery through upregulation of CXCL12 (particularly by inhibiting RGS16, CXCL12 inhibitor [30]), and miR-126* keeps endothelial proliferative reserve by targeting DLK1 [31]. Moreover, circulating levels of miR-126 were shown to be decreased in patients with coronary atherosclerosis and diabetes, which may contribute to the pathophysiology of both diseases [32, 33]. On the other hand, miR-126 transfer into VSMCs was shown to promote VSMCs turnover, thus suggesting a potentially negative role in vascular remodelling [34]. miR-126 functions thus need to be evaluated strictly in the tissue (or cell)-dependent manner.

Focusing now on VSMCs, miR-143/145 should be mentioned. To fulfil their functions in the vessel tone regulation, VSMCs need to remain in so-called *contractile* (or *quiescent*) phenotype; during various pathologies, the phenotypic switch to "synthetic" (or "proliferative") phenotype occurs interfering with VSMCs normal function [35]. miR-143/145 was shown to be one of the crucial regulators of the phenotypic switch—increased levels of miR-143/145 are needed in VSMCs to remain in "contractile" phenotype [18, 36]. Knockout of miR-143/145 leads to incomplete differentiation of VSMCs and results, e.g. in aneurysm development [36]. Interestingly, it was shown that miR-143/145 levels are increased in ECs under the laminar flow [37] and moreover, miR-143/145 is transferred from ECs into VSMCs in order to keep them in "contractile" phenotype [38]. miR-143/145 targeting thus definitely represents a potential therapeutic target in the treatment of atherosclerosis for the future.

Lastly, if talking about atherosclerosis, macrophages need to be mentioned together with miR-155. miR-155 represents a master inflammation-miRNA and the important regulator of the immune system functions [20]. It was repeatedly shown that miR-155 knockout or inhibition in experimental models of atherosclerosis led to reduction of atherosclerotic plaques size and decreased macrophage accumulation [39, 40]. Via targeting of endothelial NO-synthase (eNOS), miR-155 further disrupts endothelial relaxation, which also contributes to the pathophysiology of atherosclerosis [41]. Interestingly, circulating (particularly plasmatic) levels of miR-155 were shown to be increased in experimental models of atherosclerosis [42] and miR-155 was shown as one of the most abundant miRNAs contained within LDL particles. This may partly explain LDL proinflammatory effects [43]. On the contrary, also antiatherogenic effects or miR-155 were described—e.g. absence of miR-155 in the haematopoetic cells in the hyperlipidemic mice increased atherosclerotic plaque formation [44]. Moreover, in human subjects with CAD, circulating levels of miR-155 are reduced [32]. More information about the conflicting role of miR-155 within atherosclerosis development can be found in the recent study performed by Ma et al. [45].

miRNAs in Carotid Atherosclerosis in Humans

Human studies dealing with carotid atherosclerosis mostly determined expression of miRNAs from carotid atherosclerotic plaques obtained during endarterectomy of carotid arteries in patients with symptomatic stenosis [46–48]. Comparison of carotid plaques miRNA expression revealed a set of differentially expressed miRNAs compared to miRNA expression in normal carotid arteries and within this set, miR-21, miR-34a, miR-146a, miR-146b-5p and miR-210 were found to be upregulated [47]. In the very interesting study by Cipollone et al., the subset of five miRNAs (miR-100, miR-127, miR-145, miR-133a and miR-133b) were further shown to be increased in patients after stroke compared to patients with plaques but without stroke, thus predicting higher plaque instability [46]. In the agreement with Cipollone results, miR-145 expression was shown to be higher in carotid plaques from patients with hypertension [48], who are more prone to develop stroke if hypertension is not properly treated. Higher carotid plaque expression of miR-145 may thus be considered as a risk factor for plaque instability [46, 48]. Interestingly, higher circulating miR-145 levels were shown [49] and at the same time abolished [50] as a potential stroke biomarker (as described further).

Focusing more on circulating miRNAs, levels of miR-21 and miR-221 were shown to be higher in patients with carotid atherosclerosis [50] and urinary levels of miR-29b were shown to correlate with cIMT in patients with type 2 diabetes mellitus (T2DM) [51]. All three miRNAs may thus serve as potential markers of carotid atherosclerosis reflecting the disease severity [50, 51].

Lastly, we would like to point out very interesting role of miR-217 described in the senescence of ECs (using human umbilical vein ECs, aortic ECs and coronary ECs together with endarterectomy samples) [52]. miR-217 was shown to be expressed in human carotid plaques and it was shown to regulate the expression of silent information regulator 1 (Sirt1)—as the cells were ageing, miR-217 levels were increasing with concomitant decrease in Sirt1 levels, a phenomenon known to occur during ageing. Blockade of miR-217 reversed effect of ageing and increased angiogenic activity. miR-217 antagonism may thus represent very interesting strategy to reduce endothelial senescence and prevent the development of age-related diseases.

Summary

Both atherosclerosis in general and particularly carotid atherosclerosis represent risk factor for stroke development. Studies using endarterectomy and plasma/urine samples to reveal biomarkers of plaque instability or disease severity definitely indicate that miRNAs may serve not only as potential diagnostic but also therapeutic targets in carotid atherosclerosis. Further studies on larger cohorts and involving patients with various comorbidities are needed as well as studies using animal models of plaque instability, to reveal the true potential of miRNAs in the carotid atherosclerosis for future clinical practice.

Ischemic Stroke

Epidemiology of Ischemic Stroke

Stroke represents a major cause of disability and the second leading cause of death worldwide (after coronary artery disease), with an incidence of about 17 million per year. It affects both the elderly and the young (in 2010, 31 % of strokes affected adults under 65; more than 83,000 children and youths under 20 have had strokes). Improvements in primary prevention and lifestyle changes have led to a decreased incidence of age-adjusted stroke. Nevertheless, the overall number of strokes has been increasing and is expected to accelerate over the coming decades because of the population ageing. It is predicted that stroke will account for 6.2 % of the total burden of illness in developed countries by 2020. Without major advances in primary and secondary prevention, acute stroke management and treatment, and in post-stroke rehabilitation, the burden and cost of this disease will considerably increase [53–55]. Stroke can be classified as ischemic or haemorrhagic. Ischemic stroke can be either transient ischemic attack (TIA) or cerebral infarction. Haemorrhagic strokes can be either intracerebral or subarachnoid. Figure 9.2 shows the computed tomography (CT) differences between acute ischemic and haemorrhagic strokes.

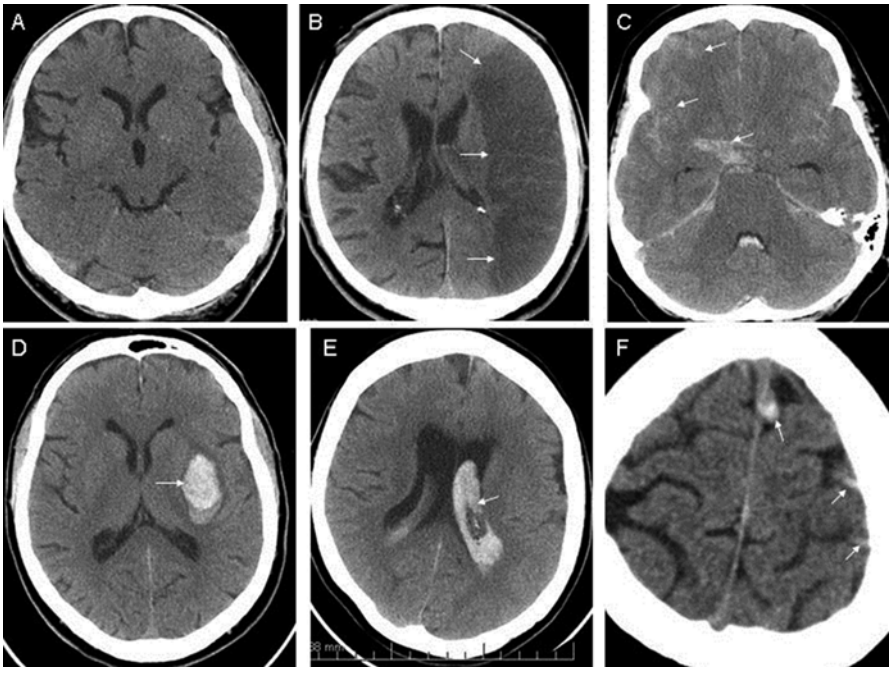

Fig. 9.2 Ischemic and haemorrhagic stroke on CT. (**a**) Normal CT (typical baseline finding in patients with acute ischemia); (**b**) acute ischemia 24 h after stroke onset (hypodense area marked by *white arrows*); (**c**) subarachnoid haemorrhage (hyperdense signal marked by *white arrows*); (**d**) intracerebral haemorrhage (ICH); (**e**) intraventricular haemorrhage; (**f**) cortical vein/dural sinus thrombosis (hyperintensities represents acute clots in cortical vein and superior sagittal sinus)

Brief Aetiology and Pathophysiology of Ischemic Stroke and Intracerebral Haemorrhage

Ischemic strokes represent at least 85 % of all strokes. Sometimes, the cerebral infarctions with a small deficit are called "minor strokes". Cerebral ischemia represents a consequence of arterial or arteriolar occlusion leading to the blood flow reduction. Possible ischemic stroke mechanisms (aetiologies) include ischemic strokes of atherotrombotic (30 %), lacunar (20 %) or cardioembolic origin (30 %); of other known aetiology, e.g. arterial dissection, vasculitis, hemodynamic infarctions, thrombophilia, cortical vein or dural sinus thrombosis (5–10 %); and 5–10 % are strokes of unknown cause termed cryptogenic strokes [54, 56].

It is important to mention that therapeutic and preventive strategies depend on the proper diagnosis of the stroke cause. Typical example is an indication for anticoagulation versus antiplatelet therapy (secondary prevention) in patients with cardioembolic versus non-cardioembolic strokes. Identification of the exact cause is also important for prognosis and treatment of additional risk factors.

Intracerebral haemorrhages (ICHs) are divided in the primary and secondary. Primary ICHs are caused by a rupture of cerebral artery/arteriole, and are typically associated with uncontrolled hypertension. Based on the localization apparent on neuroimaging methods, they can be divided into two main types: typical and atypical. The typical ICH is located in deep brain structures supplied by perforating arteries. The atypical ICHs are located more superficially (e.g. lobar haemorrhages). Atypical ICHs (20 % of all ICH) are usually secondary to medical conditions different to hypertension, such as coagulopathies, arterio-venous malformations, other vasculopathies, and tumours.

From clinical point of view, presentation of ICH is very similar to acute ischemic stroke, and therefore ICHs cannot be differentiated from acute ischemia based only on clinical examination. ICH manifestation corresponds to its location and volume, and also to the bleeding progression. Because treatments of ischemic and haemorrhagic strokes differ, brain CT or MRI are essential for differential diagnosis and treatment decision-making [57–60].

microRNA in Acute Ischemic Stroke

There are numerous roles of miRNAs in acute ischemic stroke—e.g. intracellular miRNAs participate in pathophysiology of ischemic stroke via affecting the target genes expression involved in atherosclerosis, angiogenesis and/or neuroinflammation [61]; extracellular miRNAs serve as mediators of cell-to-cell communication affecting pathophysiology and course of the disease. Molecules released into the extracellular space might represent clinically valuable biomarkers. It was shown that almost any ischemic conditions (coronary artery disease, peripheral artery disease) lead to alteration in circulating miRNA levels [62]. The tissue-specific miRNAs are released into the circulation after cell damage or death, and thus indirectly indicate the organ damage [63, 64]. Since the levels of circulating miRNAs are not random and exact mechanisms of miRNA secretion/excretion still need to be clarified, these miRNAs may even reflect the course of disease and bring to clinicians information regarding patients prognosis and outcome (e.g. in myocardial infarction, specific miRNA profile may predict development of heart failure [65]; in stroke, miRNA profiles were shown to be predictive in stroke recurrence [66], etc.).

Within the field of ischemic stroke, miRNAs have been studied both in different animal models and human stroke populations. In terms of miRNAs detection both principal approaches, i.e. studying the expression profiles of many miRNAs at the same time (“miRNA profiling”), as well as candidate-miRNAs approach (i.e. focusing more deeply on a subset of miRNAs, their possible targets and biological roles) have already been employed.

Within the following subchapter, we will focus on profiling studies giving a rationale for further miRNA studies in the field of ischemic stroke.

Animal Models of Stroke

Experimental stroke models used in following studies comprise mainly of cell culture (varying from generally accessible cell cultures such as HEK cells, N2A cells, to the primary cultured cortical neurons) of oxygen-glucose deprivation (OGD) simulating hypoxic-ischemic conditions by restriction of oxygen and glucose or pure hypoxia (e.g. for 30 to 60 min). Otherwise the H_2O_2 application responsible for simulating the reactive oxygen species (ROS) specific damage has been induced and studied. In studies focused on the roles of progenitor cells after acute ischemia, the subventricular zone (SVZ) cells has been isolated and studied in hypoxic-ischemic conditions.

In animal model studies, models of the middle cerebral artery occlusion (MCAO) have been typically used but with different approaches to the induction of occlusion (usually lasting from 60 to 120 min followed by varying periods of reperfusion and sampling). The way of MCAO technique has been also highly variable (using electro-coagulation, embolic techniques or complete mechanical obstruction, etc.). In terms of animals, spontaneously hypertensive rats (SHR), Wistar rats, Sprague-Dawley rats or C57BL/6J mice have been used.

In human studies, there is no possibility to simulate experimental stroke, thus only patients admitted to hospitals with stroke symptoms might be involved in miRNA sampling from different bodily fluids. So far, only the blood and its derivatives, and/or cerebrospinal fluid have been used in human stroke research.

Profiling microRNA Studies in Acute Ischemic Stroke

Up to date several miRNA profiling studies were performed using both animal model of stroke [67–72] and human stroke patients [73–78], in the animal studies using either brain tissue or blood or both; in the human studies using bodily fluids of patients after ischemic event.

Animal Profiling Studies

The very first miRNA profiling in animal stroke model was performed by Jeyaseelan et al. in 2008. Using MCAO model, they performed screen for 236 various miRNAs in both blood and brain at two different time points (20/106 and 25/82 differentially expressed miRNAs at 24 and 48 h in blood/brain were identified). Moreover, the expression of following miRNAs was detected for the first time in the rat brain: miR-126, miR-129, miR-129*, miR-145, miR-181a/b/c and miR-185.

Focusing on the specific group of miRNAs the authors confirmed several targets: laminin-1 and integrin-1 as targets of miR-124, VSNL1 (neuronal calcium sensor)

as a target of miR-290 (and possibly also miR-124a); aquaporin (AQP4) as a target of miR-30a-3p and miR-383, and matrix metalloproteinase 9 (MMP9) as a target of miR-132 and miR-664 [69]. In their following study, they focused on miRNAs expression in later phases of stroke (48–168 h after ischemic stroke) and identified: miR-21, miR-142-3p, miR-142-5p and miR-146a to be increased and miR-196a/b/c, miR-224 and miR-324-3p to be decreased as compared to the acute phase. Moreover, levels of miR-206, miR-290, miR-291a-5p and miR-30c-1* positively correlated with infarct volume [70].

Further, Dharap and colleagues used SHR and extended findings from the first study of Jeyaseelan by sacrificing animals at five different time points (3, 6, 12, 24 and 72 h). It was shown that 49 miRNAs were dysregulated after transient MCAO, 24 miRNAs were upregulated and 25 showed downregulation at least one of these time points. Other subsets of miRNAs were detected at two or more time points showing that expression of some miRNAs (e.g. miR-140, miR-145, miR-260, miR-292-5p) tended to increase and of other miRNAs (e.g. miR-376-5p, miR-153) tended to decrease over time, while other miRNAs were altered only transiently (e.g. miR-29c) or permanently without the specific increase or decrease (e.g. miR-344-3p).

The authors further focused on miR-145 and its target superoxide dismutase (SOD2) using antagomir against miR-145 showing that inhibition of miR-145 led to SOD2 upregulation and consequently protected the neurons from cell death [67]. Similarly to Dharap, Gubern et al. in 2013 also proved the expression change of 32 miRNAs during acute phase of stroke (30 min and 6 h) and late stroke phases (7 and 14 days). Their group further focused on miR-347 and its targets (Acsl4, Bnip31 and Phyhip) showing its importance in regulation of neuronal cell death [68].

Compared to previously mentioned studies, Selvamani et al. [71] focused on stroke from slightly different point of view—studying effect of age and sex on miRNA expression (miR-127-3p, miR-335, miR-543, miR-139-5p, miR-33a, miR-338-3p, miR-222, miR-15b, miR-92a, miR-424, miR-495 and miR-33) in blood and brain at day 2 and 5 after the MCAO. Interestingly, on the day 2, differences in miRNA levels were affected mainly by age, and on the day 5 mainly by sex showing the need to keep in mind the confounding factors.

Animal profiling studies definitely created a great background for the studies mentioned further by identifying a wide range of miRNAs altered in brain tissue after stroke. These studies showed that a large number of miRNAs was altered in rat brain after ischemic event and that this set was not completely the same as the set of blood identified miRNAs. Nowadays, it has been revealed that after stroke, also other organs than brain are involved in the complex body reaction affecting patient outcome. Thus identification of other miRNAs involved, e.g. in inflammation, or being other organ specific (e.g. finding of liver specific miR-122 to be increased in blood after stroke) may further stimulate the research in order to better understand the complex effect of stroke on the organism.

Human Profiling Studies

Unlike the animal profiling studies, where brain tissue is available and enable much deeper focus on molecular biology of stroke, only bodily fluids are available in ischemic stroke in humans for miRNA analysis.

One of the first attempts to identify circulating miRNAs in peripheral blood of 19 patients after stroke was performed by Tan et al. in 2009. Relatively young patients (aged 19–49 years) were enrolled and from 836 miRNAs studied in total, 157 were found to be dysregulated after stroke (138 upregulated, 19 downregulated). Out of these let-7f, miR-126, -1259, -142-3p, -15b, -186, -519e and -768-5p were consistently downregulated in all patients independently of the stroke subtype (according to TOAST classification).

Interestingly, miRNA clustering revealed different expression patterns in patients with good and poor clinical outcome defined as modified Rankin scale (mRS) ≤ 2 versus mRS >2. Although being a small-sized study enrolling only young individuals, the very promising results definitely identified miRNAs potentially useful as biomarkers of acute stroke and thus much larger studies were performed by the same group in 2013 [76] and 2014 [74].

In the first mentioned study, authors studies no-risk or low-risk patients with stroke, to avoid a potential effect of atherosclerosis, hypertension or diabetes on circulating miRNA profiles and identified 293 miRNAs out of whose 21 miRNAs presented with similar expression in all stroke samples [76]. Four of these, miR-25*, -34b, -483-5p and miR-498, were shown to be downregulated in all cases.

In the latter mentioned study, the authors involved two groups of stroke patients: relatively young (≈45 years) and older cohort (≈60 years) after stroke. The latter cohort displayed a number of age-related comorbidities, which also was a reason to involve healthy control (≈40 years) and patients with metabolic syndrome without the history of stroke as two control cohorts [74]. Out of 314 miRNAs studied, downregulation was observed in 58 and upregulation in 47 miRNAs in acute (24, 48 h up to 7 days) or late phase (from 2 months to 2 years) of stroke. Sixty-three miRNAs were found to be altered at both phases and five upregulated miRNAs, miR-27a*, miR-125b-2*, miR-422a, miR-488 and miR-627, showed high AUCs for patients after stroke, compared to those with metabolic syndrome. Moreover, authors showed that clustering of 32 miRNAs enabled distinguishing among individual stroke subtypes according to TOAST classification.

Another profiling after stroke was performed by Wang et al. [78]. This study group focused on early stroke detection using MRI in 136 stroke patients within 24 h from stroke onset. Based on the MRI finding, MRI(+) and MRI(−) stroke patients were distinguished and profiles were taken for these patients together with 116 healthy subjects. Altogether, 42 miRNAs were chosen for further confirmation and out of these, 13 miRNAs were confirmed as upregulated and 4 miRNAs as being downregulated. miR-106b-5p and miR-4306 showed gradual expression and being the lowest in controls, higher in MRI(−) and the highest in MRI(+) patients. Contrarily, miR-320e and miR-320d showed opposite trend, i.e. being downregu-

lated in MRI(−) and more downregulated in MRI(+) patients, thus being identified as potential biomarkers of acute stroke.

One of the very recent profiling studies of human serum was performed by Li et al. This study identified 115 differentially expressed miRNAs in ischemic stroke patients. Out of whose miR-32-3p, miR-106-5p, miR-1246 and miR-532-5p were identified as potential stroke biomarkers [79].

Another attempt to study specific roles of miRNAs in stroke was performed by Jickling et al. In this study the authors determined miRNA profiles from peripheral blood mononuclear cells, not from plasma or serum [73]. This different approach enables to study leukocytes expression profiles and reveal a potential interconnection between immunity, inflammation and stroke. In conclusion miR-122, miR-148a, let-7i, miR-19a, miR-320d and miR-4429 were decreased and miR-363 and miR-487b increased in the cells of patients with stroke and thus the potential regulation of leukocyte adhesion, extravasation and thrombi formation was proposed [73].

Most recently, Sorensen et al. published interesting paper identifying potential miRNA biomarkers of acute stroke not only from blood but also using the cerebrospinal fluid [75]. At the time of this manuscript preparation, only abstract of this study was available. The authors identified miR-151-3p and miR-140-5p to be upregulated and miR-18b-5p to be downregulated in blood after stroke and miR-523-3p was identified in more than 50 % of patients and in none of the controls in the cerebrospinal fluid. These results, together with results from other research groups mentioned above or further clearly demonstrate a potential of miRNAs as biomarkers of acute ischemic stroke and the need for complex studies taking into account not only the traditional cardiovascular and metabolic risk factors, sex, age but also using control cohorts with other neurological disorders that can resemble stroke.

Candidate microRNA Studies

Following section summarizes up-to-date information regarding animal and human studies in acute stroke with the special emphasis put on the let-7 family, miR-21, miR-29 family, miR-124, miR-145, miR-181 family, miR-210 and miR-223. Roles of other miRNAs are briefly summarized in the last subsection. Overview of below described miRNAs is provided in Tables 9.1 and 9.2, detailed description can be found further in the text.

let-7 Family

let-7 family represents a group of nine miRNAs termed let-7a to let-7i. Being one of the first miRNAs even described with important functions in *C. elegans* developmental timing [111], its functions in humans has been extensively studied and except of participating in tumorigenesis, angiogenesis and immunity, let-7 family

Table 9.1 Overview of the targets and roles of the main miRNAs involved in stroke pathogenesis

miR	Target	Model	Function in stroke	References
Let-7 family	–	–	Involved in neurogenesis and neuronal differentiation. Downregulated in stroke	[80]
	IGF-1 signalling	–	Application of antagomir against let-7f led to increased neuroprotection	[81]
miR-21	Fas-Ligand	MCAO Wistar, CN, HEK-293	Expression of miR-21 is increased after MCAO. In OGD cultured cortical neurons, miR-21 levels are unchanged. Introduction of miR-21 into HEK cells caused downregulation of Fas-Ligand. Potential therapeutic target	[82]
	Bcl-2	N2A NBLC	Increased expression of miR-21 by reoxygenation after OGD in N2A cells had antiapoptotic effect	[83]
miR-29 family	BH3-genes	MCAO, C57BL/6, hippocampal neural cells	miR-29b and miR-29c are downregulated after stroke and this can be prevented by oral supplementation of α-tocotrienol. Stereotactic injection of miR-29b improved brain function and lowered infarct size. Moreover, miR-29b transfection into cell lines protected cells from cell death	[84]
	DNMT3a	MCAO, SHR PC12 cells	MCAO in adult rat caused downregulation of miR-29c, which promotes ischemic damage to brain via DNMT1a induction. Substitution of miR-29c improves survival and neuronal damage	[85]
	Bcl2L2	MCAO, Wistar, CN	miR-29b is upregulated after ischemic insult and promotes neuronal cells death by targeting Bcl2L2 in MCAO model	[86]
	Birc2 (Bak1)	FNS in SDR	Fastigial nucleus stimulation (FNS) is known to reduce brain damage and its effect is partly mediated by downregulation of miR-29c. Application of FNS prior to ischemic insult had neuroprotective effect mediated by miR-29c	[87]
	–	MCAO, C57BL/6 J, CN	Study of miRNA expression in salvageable ischemic penumbra: *miR-29b-2*, -19b, 339-5p and miR-341 are dysregulated and they possibly participate on regulation of gene expression in this area	[88]

(continued)

Table 9.1 (continued)

miR	Target	Model	Function in stroke	References
miR-124	VSNL1	MCAO, SDR	VSNL1 expression correlated with miR-124 dynamics	[69]
	Ku70	MCAO, SDR	After 2 h of focal cerebral ischemia and 24 h reperfusion, miR-124 was downregulated and Ku70 upregulated. Intraventricular administration of miR-124 antagomir prevented neuronal cells death and decreased infarct size	[89]
	JAG1	MCAO, Wistar, SVZ NC	miR-124 levels are decreased in subventricular zone (SVZ) after MCAO and via targeting JAG-1, miR-124 affects Notch signalling pathway and reduces progenitor cells proliferation and promotes their differentiation	[90]
	Usp14	MCAO, C57BL6/N, CN	Lentiviral induced expression of miR-124 had protective effect on cortical neurons when OGD was applied and similarly, miR-124 application in animal model led to decreased infarct size and smaller functional impairment. This is mediated via miR-124-Usp14-REST pathway	[91]
	Bcl-2, Bcl-xl	MCAO, C57BL/6, SHR, CN	miR-124 levels are increased after MCAO in animal model and injection of agomir for miR-124 reduces infarct size. Pro/anti-apoptotic signalling via Bcl genes is involved in observed effect	[92]
	iASPP	MCAO, C57BL/6, N2A cells	miR-124 was increased after cerebral ischemia	[93]
miR-145	SOD2	MCAO, SHR	miR-145 is increased after transient focal ischemia, targets SOD2 and application of antagomir against miR-145 increases SOD2, however, without any significant neuroprotection	[67]

(continued)

Table 9.1 (continued)

miR	Target	Model	Function in stroke	References
miR-181	HspA5 (GRP78)	MCAO, CB57/B6, astrocytes	miR-181a is upregulated in ischemic core and downregulated in surrounding penumbra. Increase in miR-181a promotes neuronal cell death due to decrease in GRP78	[94]
	Bcl-2, Mcl-1	MCAO, SDR, N2A cells, astrocytes	Decreased miR-181a after OGD reduces apoptosis and mitochondrial dysfunction in cultured astrocytes and N2A cells. In animal model of MCAO, application of miR-181a prior to ischemic insult resulted in reduced infarcted area and prevented loss of CA1 pyramidal neurons	[95, 96]
	Bcl-2, XIAP	MCAO, CB57/B6	miR-181a antagomir treatment after stroke reduced infarction size, inflammatory response (NFκB activation, leukocyte infiltration) and improved neurological deficits in mice	[97]
miR-210	VEGF pathway	MCAO, C57BL/6	Using lentiviral vector, miR-210 was introduced to experimental animals and this caused increase in new microvessels and increase in neural progenitor cells in SVZ	[98]
	Notch pathway	MCAO SDR, HUVEC	Increase in miR-210 (using lentiviral vector) caused increased angiogenesis and thus recovery after MCAO	[99]
miR-223	AQP4	MCAO SDR	Increased expression of miR-223 correlated with decreased expression of aquaporin 4	[69]
	GluR2, NR2B	miR-223 KO mice, hippocampal NC culture	miR-223, via targeting glutamate receptors (AMPAR and NMDAR) subunits (GluR2 and NR2B, respectively) prevents glutamate-induced neurotoxicity after stroke	[100]

MCAO middle cerebral artery occlusion, *OGD* oxygen-glucose deprivation, *HEK* human embryonic kidney cells, *NC* neural cells, *NBLC* neuroblastoma cells, *CN* (primary) cortical neurons, *SDR* Sprague-Dawley Rats, *SHR* spontaneously hypertensive rats, *IGF* insulin growth factor, *Bcl-2* B-cell lymphoma 2, *DNMT3a* DNA methyltransferase 3A, *Bcl2L2* Bcl-2-like protein 2, *Birc2* baculoviral IAP repeat containing 2, *Bak1* BCL2-antagonist/killer 1, *VSNL1* visinin like 1 protein, *JAG1* jagged 1, *Usp14* Ubiquitin carboxyl-terminal hydrolase 14, *iASPP* inhibitor of apoptosis-stimulating protein of p53, *SOD2* superoxide dismutase 2, *HspA5* heat shock protein A5, *Mcl-1* myeloid cell leukaemia 1, *XIAP* X-linked inhibitor of apoptosis protein, *VEGF* vascular endothelial growth factor, *AQP4* aquaporin 4, *GluR2* AMPA receptor subunit GluR2, *NR2B* N-methyl D-aspartate receptor subtype 2B

Table 9.2 Summary of other miRNAs involved in stroke pathogenesis

miR	Target	Model	Function in stroke	References
miR-17-92	PTEN	MCAO C57BL/6 J SVZ NC	After ischemic insult to animals or cell, miR-17-92 is increased. This upregulation leads to increase in neural progenitor cells proliferation via PTEN, Wnt and c-Myc related pathways	[101]
miR-103	NCX	MCAO SDR OGD BHK, PC-12 cells, CN	Blockage of miR-103-1 with specific antagomir prevents downregulation of NCX and increases recovery and decreases infarct size in experimental animals	[102]
miR-107	GLT-1	MCAO SDR ↓O2 NG108-15 cell	Expression of miR-107 is upregulated in plasma and brain after MCAO, which downregulates GLT-1 and leads to glutamate accumulation and excitotoxicity	[103]
miR-134	HSPA12	MCAO C57BL/6 J, OGD CN	Downregulation of miR-134 reduces neuronal cell death via increase in protective heat shock protein HSPA12	[104, 105]
miR-137	Grin2A	MCAO SDR, HEK-293	miR-137 is downregulated in brain after stroke as well as after mild stress, a model of depression in rats. Upregulation of miR-137 levels restored behavioural changes, probably partially via targeting Grin2A, a component of NMDA receptor	[106]
miR-200c	Reelin	MCAO CB57/B6 H_2O_2 exp., N2A cells	Intracerebroventricular pretreatment with miR-200c antagomir results in decreased infarct size and neurological deficit, partly by targeting Reelin, protein known to be involved in neurodevelopment	[107]
miR-376-5p	HIF1α	MCAO, HUVEC	miR-376-5p is downregulated after stroke and it alters angiogenesis via affecting VEGF/Notch signalling in a HIF1α dependent manner	[108]
miR-424	CDK6, CDC25A CCND1	C57BL/6 J, murine BV2 microglial cells	miR-424 is decreased after stroke. Pretreatment and post-treatment of MCAO animals with miR-424 mimic resulted in decrease in cerebral infarction and brain edema probably via affecting microglia activation and cell cycle progression	[109]
	Nrf2	MCAO, C57BL/6 J, CN	miR-424 levels increased at 1 and 4 h and decreased 24 h after ischemia in peri-infarct cortex. By affecting levels of antioxidative enzymes, miR-424 overexpression caused protection from oxidative stress which resulted in reduced neuronal cell death	[110]

MCAO middle cerebral artery occlusion, *OGD* oxygen-glucose deprivation, *HEK* human embryonic kidney cells, *NC* neural cells, *NBLC* neuroblastoma cells, *CN* (primary) cortical neurons, *SDR* Sprague-Dawley Rats, *SHR* spontaneously hypertensive rats, *PTEN* phosphatase and tensin homolog, *NCX* sodium-calcium exchanger, *GLT-1* Glial Glutamate Transporter 1, *HSPA12* heat shock protein A12, *Grin2A* glutamate receptor subunit epsilon-1, *HIF1α* Hypoxia-inducible factors 1α, *CDK6* cyclin dependent kinase 6, *CDC25A* cell division cycle 25A, *CCND1* cyclin D1, *Nrf2* nuclear factor erythroid 2-related factor

represents one of the most abundant miRNA families in the brain, where it fulfils crucial roles in neurogenesis and differentiation, as recently reviewed [80].

In the pathophysiology of stroke, let-7 may be involved in neurogenesis [111], angiogenesis and inflammation. It is one of the miRNAs transferred via exosomes affecting the gene expression of recipient cells in the atherosclerosis process [112, 113]. Expression of let-7 family members was repeatedly shown to be downregulated in above-mentioned profiling studies [68, 69, 71], and specific blockade of let-7f using antagomir led to increased neuroprotection in the rat model via affecting the insulin like growth factor (IGF-1) signalling pathway [81]. It can be hypothesized that a downregulation of let-7 family members after the stroke leads to activation of neuroprotective mechanisms mediated at least partly by the IGF-1 signalling.

miR-21

In animal brains, miR-21expression has been reported in profiling studies as increased [69, 77] and being even more dominantly increased in small artery stroke patients [77]. miR-21 is generally known to be involved in ischemia and hypoxia related processes and also has a strong antiapoptotic effect [114].

In the study by Buller and colleagues, expression of miR-21 has been observed to be significantly increased in ischemic boundary zone as compared to contralateral neurons in the MCAO animal model. However, in OGD cultured cortical neurons, no such upregulation of miR-21 has occurred [82]. On the other hand, when miR-21 was introduced to the OGD-cells, much better survival due to decreased apoptosis via downregulation of Fas-Ligand has been proven [82]. In accordance with this, reoxygenation of the N2A cells after OGD increased miR-21 levels and also promoted N2A cells survival [83].

Taking into account that miR-21 is known to be involved not only in cerebral, but also in myocardial ischemia, where it was shown to be produced by fibroblasts in the site of infarction having antiapoptotic effect on surrounding cardiomyocytes [115], strategies leading to increased miR-21 expression may potentially serve as novel future therapeutic strategies in ischemic stroke.

miR-29 Family

miR-29 family is represented by four members: miR-29a, -29b-1, -29b-2 and 29c. With many potential targets of all family members, there is still a controversy, whether miR-29 is pro-survival or pro-apoptotic since the upregulation of miR-29b has been recognized as a neuronal survival factor interfering with apoptosis by affecting Bcl-2 and BH3 pathways [84, 116], while the downregulation of miR-29 protects hearts against ischemia-reperfusion injury [117]. Luciferase target assays have indicated that miR-29 family members target both pro- and anti-apoptotic Bcl-2 family members, and thus it is necessary to evaluate this miRNA family functions in the context of disease and model chosen in particular studies [117].

Focusing more on stroke, both the downregulation [85, 87, 116] and upregulation [86, 88] of miR-29 family members have been observed in animal MCAO models with various interpretation of observed findings. Khanna et al. and Pandi et al. reported miR-29b and miR-29c downregulated in ischemic area. This loss of miR-29b/c participated on ischemic brain damage and can be prevented by oral administration of α-tocotrienol or by re-induction of both mentioned miRNAs in the brain—all these actions resulted in decreased volume of infarct and promoted cell survival. Conversely, recently Huang et al. reported the downregulation of miR-29c after the fastigial nucleus stimulation, which is known to be neuroprotective and indeed, overexpression of miR-29c resulted in increased infarct volume and poor neurological outcomes [87]. In accordance with Huang et al., results of Shi et al. showed that miR-29b was upregulated after ischemic event and promoted neuronal cell death by targeting Bcl2L2 [86].

Slightly different approach was chosen by Dhiraj et al. [88], whose group decided to study miRNA expression in salvageable tissue called penumbra. After the three vessel occlusion followed by 24 h of reperfusion, unilateral hemispheric lesion was induced and comparison of miRNAs expressed in brain ischemic cells and cultivated neuronal cells has been performed. The miR-29b-2, miR-19b, miR-339-5p and miR-341 were found to be dysregulated in both models of ischemic damage suggesting their possible participation in the regulation of gene expression in salvageable ischemic penumbra. The authors suggested also above-mentioned Bcl-2 targeting as one of potential mechanisms [88].

miR-124

miR-124 is almost exclusivelyexpressed in the central nervous system and participates in the regulation of neuronal differentiation and adult neurogenesis [118], which makes this miRNA to be referred as "brain-specific miRNA" [119]. Already in 2009 Laterza et al. showed that brain injury (induced by MCAO) caused miR-124 upregulation in plasma, similarly to elevation of miR-122 after liver injury and miR-133 after muscle injury [119] and suggested this miRNA as possible tissue-specific biomarker. Similarly, Weng et al. using the expression profiling of 671 miRNAs in various tissues (brain, heart, lung, liver, kidney, colon, skin, skeletal muscle and pancreas) confirmed miR-124 to be preferentially expressed in the nervous system, especially in the cerebellum, cerebrum and occipital and temporal lobes, and to be present in serum 6 and 48 h after the MCAO in rats [72]. Later on, elevation of circulating miR-124 was also confirmed in the profiling study by Jeyaseelan and colleagues [69]. Moreover, they reported the visinin like 1 protein (VSNL1), a neuronal calcium sensor abundantly expressed in the central nervous system as the miR-124 target in animal stroke model [69]. Nowadays, VSNL1 is a protein studied as a potent biomarker of various dementias [120] and also miR-124 is supposed to be involved in pathophysiology of dementia by targeting β-site APP cleaving enzyme 1 (BACE1) [121]. Studies of post-stroke related neuronal damage, post-stroke cognitive decline and dementia development should definitely focus on this possible interconnection. Concerning miR-124, other numerous functions within

the brain tissue, several other targets and pathways have already been identified and are summarized in Table 9.1.

Interestingly, results concerning the miR-124 up- or downregulation after ischemic insult are contradictory, which is to our opinion caused by differences among individual studies concerning sample size, MCAO induction technique, sample collection, RNA isolation, choice of internal control and other aspects.

There are two studies reporting miR-124 downregulation after stroke in animal model of MCAO. Zhu et al. reported Ku70 (DNA repair protein), upregulation after MCAO and this upregulation may further be induced via the antagomir-124 application prior to the MCAO resulting in decreased volume of infarction [89]. A slightly different study was performed by Liu and colleagues, where the authors reported miR-124 downregulation 7 days after the MCAO. This was correlated with neuronal progenitor cells proliferation. Artificial administration of miR-124 caused decrease in progenitor cells proliferation and increase in their differentiation, thus also promoting global damage to the brain [90]. Thus, both of these studies have suggested miR-124 downregulation as a protective mechanism for brain tissue repair after stroke.

However, in two other studies, similar properties of miR-124 upregulation, i.e. preventing tissue-injury, were described [91, 92]. In the first study mentioned, miR-124 upregulation after both OGD and MCAO in cell-line and animals, respectively, were confirmed and this prevented neuronal apoptosis. Authors revealed Ups14, (de)ubiquitination-related protein, as a target of miR-124 and revealed that via Ups14 targeting, miR-124 indirectly increases levels of RE1 silencing transcription factor (REST), which upregulation was reported in neurons destined to death [91]. Thus, miR-124 application led to decreased infarct volume size and also to decreased functional impairment, determined by the Rotarod test, tight rope test and modified Morris maze test. In the latter study, miR-124 upregulation in ischemic penumbra was reported and functional studies using miR-124 knockdown/increase led to infarct volume size increase/reduction, respectively. Similarly, in the primary cortical neurons, OGD caused miR-124 upregulation and miR-124 agomir and antagomir delivery reduced or promoted cell death. These effects of miR-124 are probably mediated via Bcl-2 and Bcl-xl pathways [92].

Most recently Liu and colleagues elegantly demonstrated that miR-124 upregulation in infarcted area caused the downregulation of inhibitory member of the apoptosis-stimulating proteins of p53 family (iASPP), and thus affecting the p53-mediated neuronal cell death [93]. Compared to the aforementioned studies by Doeppner and colleagues and Sun and colleagues, who reported upregulation of miR-124, this is not beneficial for neuronal cells—blockage of miR-124 with specific antagomir caused upregulation of iASPP and this resulted in reduced infarct size and reduced neuronal cell death.

It can be concluded that miR-124 is brain-specific microRNA with plentiful roles in the regulation of neuronal cell death, apoptosis and proliferation. Since the results of so far conducted studies are contradictory, more studies are needed to confirm the exact roles of miR-124.

miR-145

miR-145 should be briefly mentioned also in the stroke field, even though it is much more connected with the vessel biology. miR-143/145 cluster is involved in the function of ECs and VSMCs; however, its levels have been reported to be upregulated in brain tissue after ischemic insult [67]. Using bioinformatics prediction, superoxide dismutase 2 (SOD2) was revealed as miR-145 target and application of antagomir against miR-145 led to SOD2 increase, however, without any significant increase in the neuroprotection [67]. Recently, miR-145 was shown to be connected to microglia function and being identified as one of the p53 targets during ROS exposition [122]. Since oxidative stress is connected with reperfusion injury, potential roles for miR-145 in oxidative stress control, exceeding its known roles in vessel biology, may come up in the future.

miR-181 Family

miR-181 family represents a highly conserved group of miRNAs that consists of four members named consecutively miR-181a, -181b, -181c and 181d, and that is highly expressed in mammalian brain [123]. Main roles of this miRNA family was reported in the hematopoiesis and inflammation [124]; however, from the molecular point of view, miR-181 family targets multiple mRNAs of proteins involved in apoptosis (e.g. Bcl-2 family of antiapoptotic proteins [125]) or in tissue protection (e.g. protective proteins of endoplasmatic reticulum or heat shock proteins [94, 126]). Ouyang and colleagues focused repeatedly on various aspects of miR-181 functions in the context of cerebral ischemia concerning regulation of oxidative stress or functions of molecular chaperons [126, 127]. In their very first study, they identified miR-181a to be upregulated in ischemic core and conversely downregulated in the surrounding penumbra corresponding with the potential of the cells to die in core and survive in penumbra [94]. Accordingly, supplementation or inhibition of miR-181 in cultured astrocytes increased and reduced astrocytes cell death, respectively, similarly to effects observed in vivo. One of the targets found to be responsible for observed effect was GRP78, one of the well-known heat shock proteins—if GRP78 levels were maintained stable, miR-181a administration did not promote the cell death [94]. Concerning further delayed neuronal cell death, miR-181 was shown to indirectly regulate the glutamate transporter (GLT-1), which induction is known to prevent the brain from excitotoxicity induced by neurotransmitter glutamate [128].

In agreement with the previous studies, application of miR-181a mimics prior [95] or after [97] the ischemic insult resulted in neuroprotection. Especially the study by Xu and collaborators has shown a great potential of miR-181 application. Since in the clinical practice, clinicians are typically faced with patients after the onset of stroke with no possibility to pretreat them. Xu and colleagues applied miR-181 antagomirs either intravenously (1 h after the MCAO) or intracerebroventricularly

(2 h after the MCAO) and interestingly both ways of application have shown reduced infarction volume (at about one third at 48 h after reperfusion). Neurologic deficit was assessed by the Rotarod test and was significantly improved as well as the overall 4-weeks survival (90 % versus 72 % in sham) and neurobehavioural recovery. In conclusion, miR-181 antagomir treatment showed great promises for the future therapeutic applications in the early post-stroke treatment.

miR-210

miR-210, a master hypoxia-miR, represents a hypoxia-induced and anti-apoptotic miRNA with approximately 35 specific transcripts as its direct or indirect targets [129]. Its overexpression in normoxic human endothelial cells stimulates formation of capillary-like structures, and increases cells migration and differentiation [130]. Consistent with the reported induction under hypoxic conditions via HIF-dependent and independent pathways [129], miR-210 expression was reported to be higher in ischemic brain, where it plays many protective roles against ischemia induced injury [98, 99]. In the setting of cerebral ischemia in rat or mice, increase in miR-210 by lentiviral vector led to increased number of new vessels [99] and progenitor cells in subventricular zone [98], thus resulting in increased angio- and neurogenesis, and preventing mice from ischemia-induced injury. To sum up miR-210 represents another promising target for stroke therapeutics.

miR-223

From the more deeply studied miRNAs, as the last one miR-223 will be mentioned. Together with miR-130a and miR-320, this miRNA was reported to target aquaporin genes, especially aquaporin 4 that is typically expressed in the brain [131]. miR-320a was shown to directly affect the expression of aquaporin 4 and aquaporin 1. Blockade of miR-320a with antagomir led to increase in the aquaporin expression and reduction of infarct volume [131]. Similar results were obtained with the use of antagomir-130a. Reduction in infarct size and increased neuronal recovery were observed after the application [132].

miR-223 is of further interest, since except of its proved aquaporin targeting [69], it was also reported as a platelet-secreted [133] and lipoprotein particle-transferred [134] miRNA, playing one of the central roles in the cholesterol metabolism [135]. Similarly to miR-181 mentioned above and miR-107 mentioned further, miR-223 also has a potential to protect brain from glutamate excitotoxicity by targeting the glutamate receptor subunits GluR2 and NR2B [100]. Moreover, in the study by Duan et al. it was observed that miR-223 levels were lower in patients with diabetes mellitus, which promoted platelet activation and increased the risk of stroke development in DM patients [136].

Other miRNAs in Ischemic Stroke

From the profiling studies mentioned at the beginning of the chapter it is obvious, that more miRNAs are involved in the pathophysiology of acute stroke than those described above. However, information regarding their function still need to be determined or is of a limited extent, thus we provide only basic information regarding their pathophysiologic, diagnostic or therapeutic potentials.

Due to its involvement in general cellular processes, miR-17-92 cluster has been described to be involved in the pathophysiology of almost any disease [137], including stroke [101]. In the subventricular zone of animals after MCAO, miR-17-92 cluster levels were increased and this, via targeting of PTEN, increased a proliferative capacity of neural progenitor cells, as confirmed also in vitro.

Following the numerical order, other miRNA involved in stroke is miR-103. It is responsible for a regulation of Na^{+}–Ca^{2+} exchanger (NCX). Its downregulation by a specific antagomir reduced brain damage and neurological deficit in experimental animals [102]. Like above-mentioned miR-181 family, also other miRNAs were shown to affect the glutamate signalling (particularly miR-107 [103]) or target heat-shock proteins (particularly HSPA12), e.g. miR-134 [104, 105]. Another miRNA recently described to promote stroke damage is miR-200c. Its blockade increased levels of Reelin, thus promoting neuronal migration and synaptogenesis, resulting in neuroprotection [107]. Concerning angiogenesis, similar effects to miR-210 were observed in miR-376-5p. This miRNA affects the VEGF/Notch signalling modulating angiogenesis as well [108].

Last but not the least, miR-424 was shown to be downregulated in brain tissue within the profiling studies [69] and it was also shown to be downregulated in peripheral leukocytes in patients after stroke [109, 110]. Restoration of miR-424 activity led to decrease in infarct volume and neuronal cell death, which was associated with decreased microglia activation [109] and reduced oxidative stress [110].

Candidate microRNAs as Biomarkers of Acute Stroke

Using animal models, elevation of serum miR-124 was reported in two independent studies and this upregulation was consistent for at least 48 h after stroke induction [72, 119], which would make miR-124 as a potential stroke biomarker. However, no correlations with outcome of experimental animals or infarct size were observed [72, 119]. Interestingly, in human studies, levels of miR-124 were reported both to be upregulated [138] and downregulated [139] after acute stroke. In the study by Leung et al., upregulation of miR-124 was prominent only in the early phase (below 6 h). In the further sampling, levels of miR-124 were significantly lower compared to acute phase [138]. Leung et al. moreover showed that miR-124 levels were higher in patients with haemorrhagic stroke compared to ischemic stroke only [138]. In another study by Liu et al., miR-124 levels were downregulated and they negatively correlated with infarct volume, CRP levels and levels of matrix metalloproteinase 9

(MMP9), thus somehow reflecting damage of neural tissue and inflammatory activity. However, no correlation with stroke severity defined by NIHSS was observed [139]. Similar properties were observed also for another neuri-miR, miR-9. The true diagnostic potential of miR-124, although being termed as brain specific, needs to be determined in larger multicentric studies since the results of currently conducted studies are contradictory.

Leaving miR-124 and moving further with human studies, let-7, miR-21, miR-30a and miR-126, miR-221 levels were studied as potential diagnostic and prognostic biomarkers.

Concerning let-7, miR-30a and miR-126, altogether 247 patients at different time points after stroke (in the acute phase within 24 h, in the subacute phase within 1 week and in the chronic phases within 48 weeks after stroke) were enrolled. It was observed that let-7 levels were decreased after stroke with a slow restitution after the 24th week. Interestingly, levels of let-7 were more profoundly decreased in patients with stroke of atherosclerotic aetiology [140]. Cut-off value for the let-7 was calculated to 1.675 at 24 h with the sensitivity 92 % and specificity 84 %.

Another larger study was performed in 233 patients (partly patients with ischemic stroke and partly patients with carotid atherosclerosis without ischemic stroke) and 157 controls focusing on miR-21, miR-221 and miR-145 levels in the serum after ischemic stroke [50]. miR-21 and miR-221 showed opposite trends, i.e. miR-21 being upregulated and miR-221 being downregulated in patients compared to the controls. Concerning miR-145, its levels were not detected in more than 50 % of study subjects and its potential for stroke diagnosis is thus not very promising [50]. However, if the whole blood RNA was isolated, miR-145 levels were shown to be upregulated after ischemic stroke in a smaller study by Gan et al. [49] and also in the profiling study performed by Sepramaniam et al. [74]. Gan et al. moreover suggested increase in miR-145 to be predictive for better outcome, due to miR-145 targeting of KLF-4/5 and its effect on reendothelization [49].

Promising results were observed for miR-210, previously mentioned as hypoxia-miR, which levels were reported to be downregulated in peripheral blood leukocytes in patients after stroke. Decrease in miR-210 levels in these patients was continuous and deepened within 2 weeks from stroke onset [141]. Moreover, after the correlation with NIHSS and mRS, patients with higher miR-210 levels had better prognosis, compared to patients with the lowest miR-210 expression. Zeng et al. further determined miR-210 peripheral blood mononuclear cells (PBMC) levels together with the levels of other potential biomarkers, including various cytokines (e.g. IFNγ, IL-1, IL-6, TNFα and others) and haemostatic markers (fibrin and its degradation products FDP, D-dimers, etc.). Combination of miR-210, FDP and IL-6 had even higher sensitivity and specificity for stroke outcome prediction (defined by mRS after 3 months) compared to the use of individual markers alone [142].

Last but not the least, also miR-223 was suggested as a potential biomarker of acute stroke [143]. After dissociation of erythrocytes, RNA was isolated from the remaining cells and miR-223 levels were determined to be increased after stroke with more prominent increase in large and small vessel stroke subtypes. Moreover, a negative correlation with NIHSS (and in animal part of the study with infarct size)

and a positive correlation with circulating IGF-1, one of miR-223 targets, were observed. Due to its pleiotropic effect, miR-223 specificity for stroke needs to be tested in other consecutive studies.

Recently, Kim et al. focused on atherosclerosis related miRNAs in the diagnostics of acute stroke. They revealed that miR-126 levels correlated with brain atherosclerotic damage and that levels of miR-17 were increased after acute stroke being also predictive of early stroke recurrence (within the study period, thus probably 1 year—only abstract was available to authors during the manuscript preparation [66]).

Future Directions

In conclusion we would like to point out the tremendous development and progress that has been done regarding the roles of miRNAs in ischemic stroke. Studies above are definitely informative about the involvement of miRNAs in the stroke pathophysiology and also about their potential clinical utilities as diagnostic, prognostic and even therapeutic tools.

For future research, it is needed to define the standardized protocols of miRNAs detection, extraction and isolation. Current studies differ and vary a lot in the source of miRNAs—in animal studies, sometimes ischemic core and sometimes penumbra have been used and this has not been quite often precisely specified; in the human studies, miRNA source ranged from the whole blood through plasma or sera to the isolated cells only. Also, various internal controls are used, sometimes using additive cel-miR-39, sometimes using miR-16 or another defined ubiquitous miRNA. Universal internal control should soon be established to normalize the data from various studies and obtain clearer and more normalized results. Also the need of multicentric studies employing more than 300 subjects is anticipated and this inquires specific needs of proper and universal sample storage protocols.

Last but not the least, the miRNA potential in stroke therapy should be further investigated and focused not only on effects of injected ago- or antagomiRs, but also determining potential side effects, mechanisms of uptake and elimination. Also, the ways of application should be precisely defined in order to increase specific targeting of brain tissue.

Intracranial Aneurysms

Epidemiology of Intracranial Aneurysms

Intracranial aneurysms represent acquired lesions of cerebral arterial wall occurring in 0.5–3 % of general population and accounting for 80–85 % of non-traumatic subarachnoid haemorrhages (i.e. bleeding into subarachnoid space).

These saccular-shaped lesions are typically localized in proximal arterial bifurcations. Typical anatomical localizations are as follows: internal carotid artery (ICA), anterior communicating artery (ACom), middle cerebral artery (MCA), tip of basilar artery (BA) and arteries of the cerebellum.

Risk factors associated with higher occurrence include higher age, female sex, cigarette smoking, hypertension and genetic predisposition (e.g. autosomal dominant polycystic kidney disease, Marfan's syndrome, Ehlers-Danlos syndrome type IV) [144–147].

Clinicians are faced with a decision regarding an optimal management of intracranial aneurysms; however, there are still many unsolved controversies regarding the risk of rupture and treatment of smaller asymptomatic aneurysms due to lack of randomized control trials. Decisions are thus made regarding patient history data, size and location of aneurysm/s, expert opinion and guidelines.

On the one hand, the particular effect of aneurysms growth on subsequent risk of rupture is not determined; on the other hand, patients with obvious enlargement should be strongly considered for interventional treatment (endovascular coiling or neurosurgical clipping, Fig. 9.3 [148]).

For those treated conservatively, proper management of hypertension, smoking cessation and repeat CT angiography or MR angiography are strongly recommended (Fig. 9.4) [144].

Biomarkers predicting the clinical course of intracranial aneurysm are still lacking. As long as miRNAs represent potential biomarkers sensitively reflecting the diseases state, maybe their potential role in the monitoring of aneurysms will come up in the near future.

microRNA Profiling Studies and Aneurysms

Animal Studies

Up to date there is only one study using animal model performed by Lee et al. in 2013 [149]. Aneurysms formation was induced by ligation of the left common carotid artery and the posterior branches of both renal arteries in Sprague-Dawley rats. Biological sampling from aneurysms (dissected out of left posterior communicating artery) or from healthy arteries (dissected out of the circles of Willis) were obtained and subsequent miRNA array profiling was done identifying 14 miRNAs upregulated (miR-1, miR-21, miR-22-5p, miR-24-1-5p, miR-26b, miR-29a, miR-29b, miR-29c, miR-101b, miR-140, miR-147, miR-181c, miR-223, miR-451) and 6 miRNAs downregulated (miR-92b, miR-138, miR-181d, miR-433, miR-489, miR-551b) in aneurysmatic vessels. Detected miRNAs are known to be involved in inflammation, apoptosis or angiogenesis and more detailed studies are definitely needed to validate the profiling results and to better understand the intracranial aneurysms development.

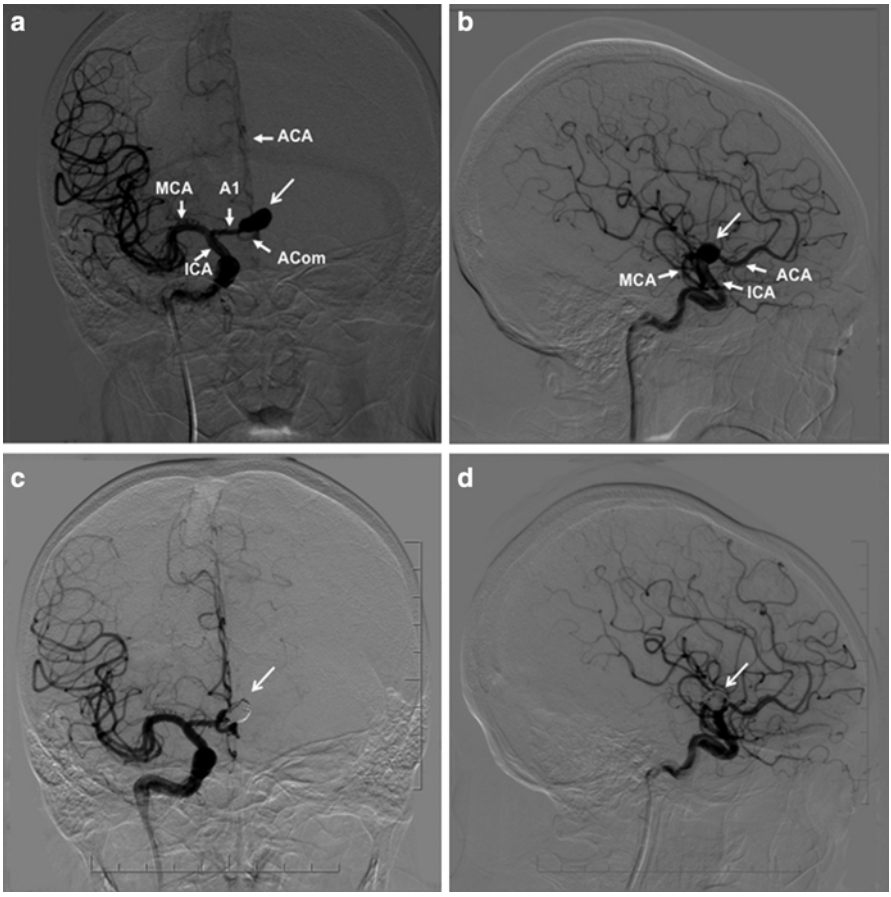

Fig. 9.3 Endovascular coiling of ruptured anterior communicating artery aneurysm, digital subtraction angiography images (DSA images). Seventy-five-year-old female with a ruptured aneurysm as a source of subarachnoid haemorrhage indicated for interventional treatment. (*top*) Subtracted right internal carotid artery angiograms in antero-posterior (*top left*) and lateral (*top right*) view showing aneurysm (*open arrow*) of the anterior communicating artery of size 12×6 mm (*bottom*). Final control series after the endovascular coiling showing almost total obliteration of the aneurysmal sac. *A1* A1 segment of the anterior cerebral artery, *ACA* A2 and A3 segments of the anterior cerebral artery, *ACom* anterior communicating artery, *ICA* internal carotid artery, *MCA* middle cerebral artery

Human Studies

Unlike in the case of stroke in human studies biological material other than bodily fluids can be collected when the intracranial aneurysms (IAs) are surgically treated. So far, there were two profiling studies using tissue obtained from patients during the surgery and two studies focusing on detection of intracranial aneurysms using circulating microRNAs.

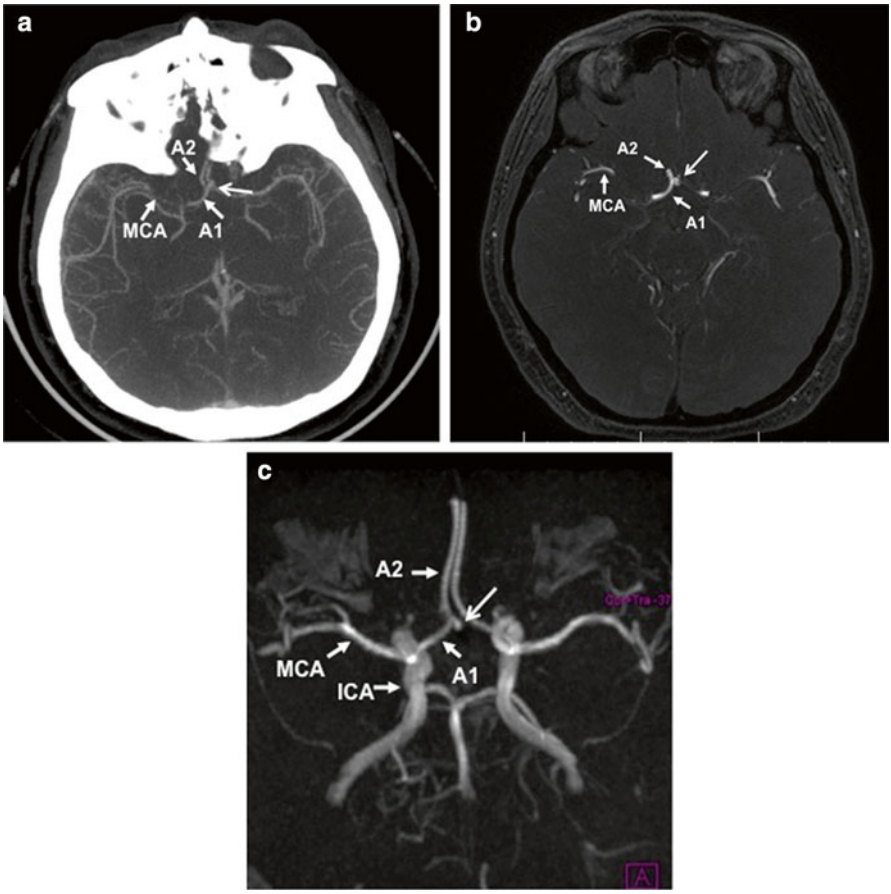

Fig. 9.4 Management of residual aneurysm sac after endovascular coiling. Forty-two-year-old female with anterior communicating artery aneurysm indicated for endovascular coiling. A tiny residual aneurysm was revealed on MRI control scanning 6 months after the procedure (conservative approach with regular MRI controls was recommended). (*top left*) CT angiography showing the aneurysm (*open arrow*) of the right anterior communicating artery (*top right*) MR angiography revealing a residual filling of the aneurysm (*open arrow*), size of 2 mm (*bottom*) MRA angiography reconstruction of the circle of Willis. *A1* A1 segment of the anterior cerebral artery, *A2* A2 segment of the anterior cerebral artery, *ICA* internal carotid artery, *MCA* middle cerebral artery

In the study by Jiang et al. (2013) microarray analysing of full-thickness vessel wall samples from 14 ruptured IA patients and 14 controls was made. Thirty differentially expressed miRNAs were found to be dysregulated and 18 miRNAs were found to be significantly different between IAs and control group (miR-1, miR-7-1-3p, miR-23b-3p, miR-24-1-5p, miR-28-5p, miR-28-3p, miR-29b-2-5p, miR-29c-5p, miR-29c-3p, miR-133a, miR-133b, miR-140-3p, miR-143-5p, miR-143-3p, miR-145-5p, miR-145-3p, miR-455-5p) [150]. One year later, Liu et al.

screened IA samples and found 157 differentially expressed (72 upregulated and 85 downregulated) miRNAs. Out of these, let-7a, miR-1, miR-30c and miR-101 were shown to be involved in the regulation of programmed cell death, extracellular matrix organization response to oxidative stress, TGF-β signalling pathway and smooth muscle proliferation [151].

Profiling studies focusing on circulating miRNAs were performed by Jin et al. in 2013 [152] and Lie et al. in 2014 [153]. In the study by Jin et al., blood samples of 24 IA patients were analysed. Patients were divided into four groups according to aneurysm characteristics (including aneurysms with/without daughter aneurysm, ruptured aneurysms and angiography-negative group). Aneurysms were located in the ophthalmic artery, basilar artery, posterior communicating artery and internal carotid artery. Altogether 86 miRNAs (69 upregulated, 17 downregulated) were identified by microarray study. In the larger study by Li et al., authors included 40 IA patients (20 with ruptured IA and 20 with unruptured IA) and 20 healthy volunteers (having neurological symptoms, such as headache, indicated for digital subtraction angiography) [153]. One hundred nineteen miRNAs were significantly changed in patients with unruptured aneurysms and 23 miRNAs in patients with ruptured aneurysms (20 of them in both ruptured and unruptured patients). miR-16 and miR-25 were studied in more detail, because these miRNAs were the most abundant in plasma. Using logistic regression, authors proved hypertension and levels of these two miRNAs, as independent predictors for the presence of IAs.

Candidate miRNA Studies and Intracranial Aneurysms

miR-21

miR-21, as described in previous chapters dealing with stroke, has very important roles in hypoxia, apoptosis and tumorigenesis. Role in the aneurysm formation was suggested in 2012 in the study by Maegdefessel et al.—authors showed increased miR-21 expression in the developed abdominal aortic aneurysm and this upregulation seemed to be protective since miR-21 blockade caused aneurysm enlargement [154]. Shortly after this pioneering attempt, potential roles of miR-21 were also shown in IAs in profiling studies observing also increase in miR-21 levels suggesting a potential regulatory role for miR-21 in controlling aneurysmal growth [153, 154].

miR-26

miR-26 is known to be involved in programmed cell death and response to oxidative stress, TGF-β signalling pathway and importantly in smooth muscle cell proliferation—it is one of the miRNAs involved in the regulation of phenotypic switch of VSMCs through Smad1 and Smad4 proteins [155]. Within the rat model of abdominal aortic aneurysm, levels of miR-26 were found to be decreased, suggesting a

potential failure in the regulatory mechanism, since inhibition of miR-26 expression should promote apoptosis and differentiation [155]. On the contrary, in profiling studies both Lee et al. and Li et al. observed increased level of this miRNA in IA rats and patients, respectively [149, 153]. Distinct roles of miR-26 in aneurysm formation thus still needs to be clarified.

miR-29 Family

All three members of miR-29 family (miR-29a, miR-29b and miR-29c, see above) have been associated with aneurysm formation, as shown mostly on abdominal aortic aneurysms—miR-29 inhibition attenuated and miR-29 overexpression promoted abdominal aneurysm formation in various animal models [156–158]. Concerning IAs, contradictory results are currently present. While two profiling studies revealed that levels of miR-29b are robustly downregulated in IA [150, 151], other two indicated miR-29 upregulation [149, 153]. Since miR-29 is known to be involved in the formation of extracellular matrix and fibrotic processes, its potential involvement in aneurysm formation should be definitely studied into more detail [159].

miR-143 and miR-145

Considering miR-143/145 function in the VSMCs, it is not surprising that it has been studied in the field of intracranial aneurysms. In the profiling performed by Jiang et al., miR-143/145 levels were found to be decreased [150]. Elia et al. further focused on miR-143/145 functions in the development of IAs and also detected downregulation of miR-143/145 levels [36]. Downregulation of this cluster is known to promote VSMCs phenotypic switch to "proliferative" phenotype, which may theoretically results in aneurysm formation, however, since the whole research discovering the roles of miRNAs in IAs is at the beginning, more functional studies are still needed to describe precise mechanism leading to IA formation.

Brain Arteriovenous Malformations

Epidemiology of Brain Arteriovenous Malformation

Brain arteriovenous malformations (AVM) represent a complex (nidus) of abnormal arteries and veins that directly fistualize without an intervening capillary bed (Figs. 9.5 and 9.6). They differ in morphology, size, location, particular clinical symptoms and risk of rupture (and subsequent intracranial bleeding). Narrow incidence is estimated to 1.12–1.42 cases per 100,000 person years.

The first-ever haemorrhage at presentation range from 38 to 68 %. This clinical symptom represents the most common one followed by seizures and/or headache. Nevertheless, the AVMs natural history and risk of rupture is still largely unknown [160–163].

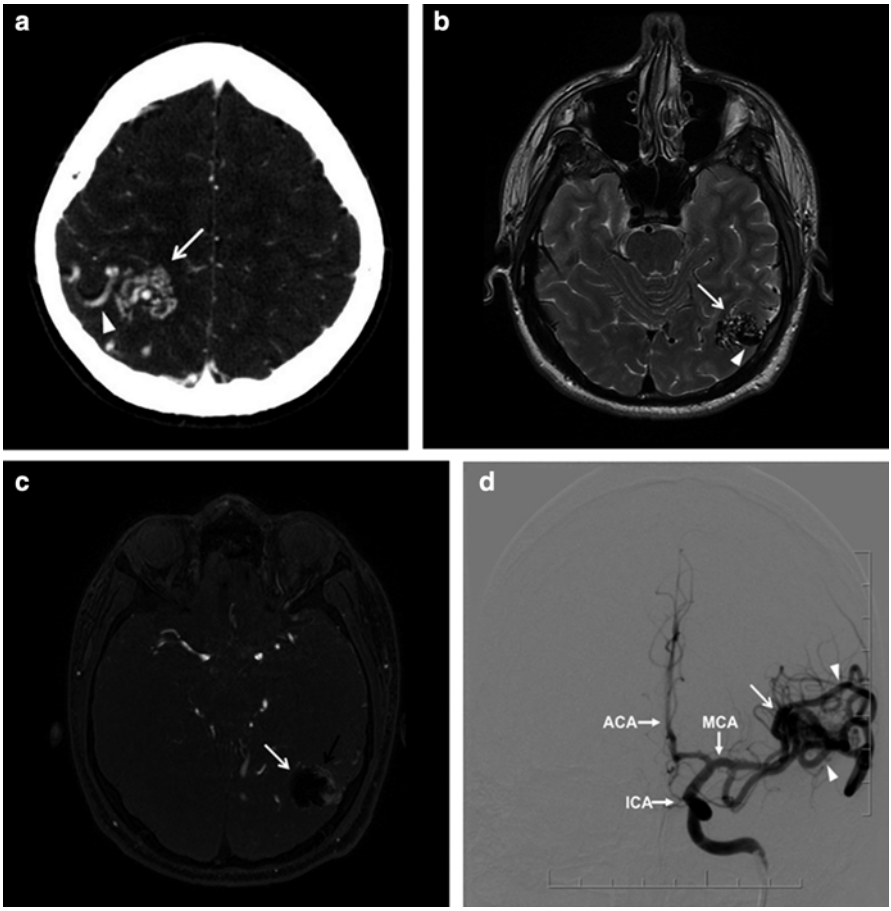

Fig. 9.5 Diagnostic imaging of brain arteriovenous malformations. (*top left*) CT angiography, scan showing a hyperdense mass of enlarged feeding arteries (*arrow head*) supplying the nidus (*open white arrow*) (*top right*) MRI, T2 weight image. The nidus appears on the scan as a hypointense mass with a feeding artery (*bottom left*) MR angiography, scan demonstrating a residual filling (*open black arrow* pointing to the hyperintense area) of the nidus after endovascular embolization of AVM (hypointense region marked with *white open arrow*) (*bottom right*) Digital subtraction angiogram demonstrates a blood supply (feeding arteries) of the AVM (*open white arrow*) from branches of the middle cerebral artery (*arrowheads*)

AVM rupture and subsequent intraparenchymal bleeding account for the most devastating complications with overall annual rate of haemorrhage for non-treated malformations 2.1–4.1 %. Based on several multivariate analyses, the nidus size (large nidus is more predictive of increased risk for future bleeding and re-bleeding), deep brain location and deep vein drainage were determined to be significant in risk for spontaneous haemorrhage [164–166].

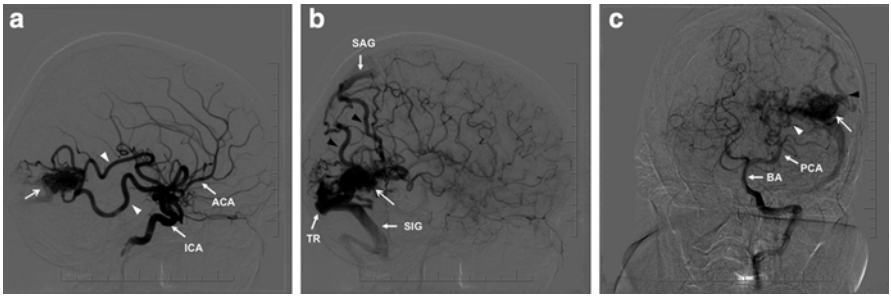

Fig. 9.6 Arteriovenous malformation on digital subtraction angiography (DSA). DSA represents a gold standard in a diagnostic management of AVM. It is widely used to delineate location, number of feeding vessels and pattern of drainage. AVM on the angiogram appears as a packed mass of enlarged feeding arteries (*white arrow heads*) that supply the central nidus (*open arrow*). Scans present a 19-year-old patient with AVM in the parieto-occipital region. The nidus is fed by vessels from carotid artery and vertebrobasilar circulation. Dilated veins (*black arrow heads*) drain the nidus into the superior sagittal sinus and transverse sinus. Abnormal venous opacification in arterial phase of DSA represents evidence for pathological shunting; (*left*) Lateral view of the AVM showing feeding arteries arising from branches of the middle cerebral artery (anterior circulation); (*middle*) Lateral view showing draining veins into the superior sagittal and transverse sinus; (*right*) Anteroposterior view showing feeding arteries arising from the posterior cerebral artery (vertebrobasilar circulation). *ACA* anterior cerebral artery, *BA* basilar artery, *ICA* internal carotid artery, *PCA* posterior cerebral artery, *TR* transverse sinus, *SAG* superior sagittal sinus, *SIG* sigmoid sinus

Studies regarding angio-architecture features (regarding muscular and elastic laminae and presence of intervening brain parenchyma), risk of rupture and AVM evolution are needed in order to help clinicians in treatment decision-making and management.

miR-18a and Cerebral AVMS

Information regarding the role of miRNAs in AVMs are limited to miR-17-92 cluster and out of this cluster almost entirely on miR-18a. As mentioned before, miR-17-92 cluster is known to play multiple functions in almost any tissue [137]. miR-18a represents an important member of miR-17-92 cluster which is involved in the regulation of angiogenesis. Antiangiogenic activity in AVM progression was particularly influenced by miR-18a (together with miR-17, miR-19a and miR-20a). Overexpression of miR-18a and miR-19a regulated the levels of thrombospondin 1 and the connective tissue growth factor (CTGF) [24, 167, 168].

Furthermore, in 2013 Ferreira et al. used AVM samples from six patients in order to determine the effect of miR-18a introduction on AVM-derived brain endothelial cells (AVM-BECs). miR-18a introduction led to increase in TSP-1 and decrease in inhibitor of DNA-binding protein 1, which resulted into better AVM-BECs function suggesting miR-18a supplementation as a potential AVM treatment [168].

Conclusions

Determining the roles of microRNAs in cerebrovascular diseases is still at its beginning; however, much progress in the field has already been done and much progress is yet to be expected. Better understanding of the post-transcriptional regulation of gene expression in endothelial cells, vascular smooth muscle cells, fibroblasts or even neurons or glia may direct further research and development of new drugs based on RNA interference for the treatment of atherosclerosis, stroke or its complications. Studies focusing on circulating miRNAs may reveal new and unexpected communication pathways among individual tissues and these findings may represent a potential source of new biomarkers, reflecting organ damage, or being predictive of the response to therapy or patients prognosis. We can definitely sum up that miRNA research hold great promises for the future therapeutics, diagnostics and for promotion of personalized medicine.

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