Editorial

Shades of microRNAs: new biomarkers in acute ischemic stroke?

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Comment on: Tiedt S, Prestel M, Malik R, *et al*. RNA-Seq Identifies Circulating miR-125a-5p, miR-125b-5p, and miR-143-3p as Potential Biomarkers for Acute Ischemic Stroke. Circ Res 2017;121:970-80.

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Acute ischemic stroke (AIS) is a major worldwide life-threatening pathology

AIS is a thrombotic syndrome, which is the result of a reduced or an interrupted blood flow to the brain, culminating in cell death, whereby approx two million neurons and 14 billion synapses are lost during each minute of stroke. According to the World Health Organization, 15 million people worldwide suffer stroke each year, and of these, 5 million die and another 5 million are permanently disabled as a result. Thus, stroke is the leading cause of long-term disability and ranks second after ischemic heart disease as a cause of death worldwide. Although the incidence of stroke is on the decline in developed countries, largely due to efforts to lower blood pressure and reduce smoking, the overall rate of stroke is increasing exponentially due to an ever-aging population (1).

In Western societies, about 80–85% of AIS are mediated by focal cerebral ischemia due to arterial occlusion by a thrombus that prevents oxygen supply to the brain, whereby 15–20% of cases are caused by leakage or rupture of blood vessels thereby provoking hemorrhagic stroke. If the blood flow to a portion of the brain is partially blocked and disability symptoms only last for a short period of time, this "mini-stroke" is known as a "transient ischemic attack" (TIA). Arterial occlusion in AIS is most commonly embolic: either cardio-embolic, from causes such as atrial fibrillation or valvular heart disease, or arterio-embolic, from atherosclerotic plaque rupture in the extracranial cervical carotid or vertebral artery. In contrast, acute coronary syndromes are mainly due to the rupture or erosion of an athero-sclerotic plaque, followed by *in situ* thrombus formation as a typical wound-healing response, which in turn causes arterial obstruction (2). In any respect, there is a very large gap between the vast number of stroke patients with the adverse consequences for public health worldwide and the lack of appropriate measures for diagnosis and prognosis in AIS.

While the main risk factor for AIS is high blood pressure, other predetermined conditions such as smoking, obesity, high blood cholesterol, diabetes mellitus, and atrial fibrillation are risk situations as well and contribute to the onset of disease. Diagnosis is typically performed with computer-tomography or magnetic resonance imaging scans along with a physical examination and blood tests to determine any risk factors and rule out other possible causes. Yet, only in about half of the AIS patients can any abnormalities be detected using these imaging techniques. AIS is an emergency situation for the affected patient as well as for the healthcare team in charge: This not only calls for a fast treatment modality, currently using fibrinolytic agents such as tissue plasminogen activator (t-PA), to remove the thrombus as "time is tissue", but it also requires safe diagnosis and prognosis options which are equally important and urgently needed (3). Despite the fact that AIS is an unpredictable event in the majority of cases, and up to 40% of all strokes are cryptogenic, specific (prognostic) blood biomarkers for AIS are currently not available. Here, a recent report in *Circulation Research* (4) which promotes circulating microRNAs (miRNAs) as potential biomarkers for AIS, may re-address this situation.

Circulating miRNAs as biomarkers for disease

Since the identification of the first small non-coding RNA

in 1993, our knowledge about miRNAs has vastly increased, and their potential use as markers of disease diagnosis and prognosis, and as new drugs or therapeutic targets has been developed in several fields of medicine (4). miRNAs are short (20–25 nt) non-coding RNAs that regulate gene expression at the post-transcriptional level by affecting both the translation and stability of miRNAs. miRNAs are also released into the extracellular milieu by many cell types, including platelets, predominantly with the help of micro-vesicles, or in association with RNA-binding proteins to protect the miRNAs against degradation by RNases. Using this horizontal transfer route, circulating miRNAs can thus be taken up by target cells where they may trigger post-transcriptional regulation as well. Due to their remarkable stability in body fluids and the relative ease of detection, circulating miRNAs appear to be the ideal tools for rapid and non-invasive diagnosis if their extracellular levels can be associated or correlated directly or indirectly with the cause or consequence of the disease or the pathological situation.

In particular, a multitude of miRNAs are expressed in the cardiovascular system, and a growing body of experimental evidence supports the significant contribution of miRNAs in the pathogenesis of neo-intimal lesion formation, atherosclerosis or ischemic heart disease (5-10). Although platelets are anucleate blood cells and are no longer able to generate RNAs *de novo*, they are major contributors to cardiovascular disease development and thrombus formation. Yet, they contain a large number of RNA species (inherited from megakaryocytes), including premature and mature miRNAs, which are released upon conditions of platelet activation and aggregation (11-13). Nevertheless, for the purpose of diagnosis, miRNA measurements need to be standardized in and across different patient cohorts also by ruling out or minimizing confounder effects; the miRNA stability in samples may be increased by adding RNase inhibitor, and preparation of plasma or serum samples is a critical issue due to the contribution of platelet-derived miRNAs in the latter one.

Circulating miRNAs as biomarkers for AIS

In their comprehensive investigation, Tiedt *et al.* (3) reported on the identification of three differentially regulated circulating miRNAs after ASI, using RNA sequencing for discovery and subsequent validation and replication of results in different patient cohorts, and further combining these data with experimental studies in animal models. The self-critical analysis of this step-wise approach included a relatively small group of 20 ASI patients (*vs.* 20 healthy subjects) for discovery, 40 patients and 40 controls for validation, and a larger group of 200 ASI patients and 100 controls for replication. Together, miRNAs 125a-5p, 125b-5p and 143-3p were singled out from this elaborated approach and were proposed as potential biomarkers for ASI. Moreover, longitudinal analysis of miRNA levels revealed that all three miRNAs showed maximal levels during the first week following the event (particularly on day 1), but not after 90 days. Interestingly, the authors found correlations between elevated miRNA levels and large vessel stroke, cardio-embolic stroke and AIS of undefined pathogenesis, but no correlation with infarct volume. Using *ex vivo* experiments with isolated platelets they further claim that platelets are a major source of the identified miRNAs, thereby contributing to the increase of these biomarkers in patient blood plasma. Although this study provides new and comprehensive aspects as to the use of circulating miRNAs in the diagnosis of AIS, which lacks appropriate protein or low molecular weight biomarkers, a number of issues need to be clarified and resolved before such a diagnostic approach can enter clinical practice.

In an editorial by Karakas and Zeller (14) in association with the original report, constructive comments were raised that should be taken into consideration by follow-up investigations in this area. The issues include the following aspects: (I) larger cohorts with less selected groups need to be studied to reflect the real-world scenario of non-selected patients, including independent and prospective sample analysis; (II) further efforts in the standardization of miRNA analysis need to be accomplished, since due to various causes of stroke, the repertoire of differentially regulated and circulating miRNA may differ considerably, calling for normalization strategies to avoid the generation of data artifacts. It remains totally unclear at present, whether the three indicated "platelet-derived" miRNAs are really of megakaryocyte origin or derived from other cellular sources and then become taken up by blood platelets during their life-time. Thus, the clarification of causal relationships between such miRNAs and different predetermining situations for AIS should be characterized experimentally; (III) although in the present report a certain biomarker signature of three miRNAs was put forward, it remains to be demonstrated whether this or another signature (including non-miRNA biomarkers) may be applicable under daily routine clinical conditions. Moreover, what is the outcome of biomarker analysis in those patients who lack abnormalities on admission computed tomographic

scans, currently the gold standard for diagnostic evaluation?

Perspectives

Additional mechanistic insights for the indicated three miRNAs may come from studies on their expression and regulation in disease, whereby (I) miRNA-125a-5p was previously shown to be associated with deleterious effects of ox-LDL on brain endothelial cells (15), (II) miRNA-125b-5p expression demonstrated associations with classical risk factors for coronary heart disease (16), and (III) miRNA-143 appears to be a predictive factor for in-stent restenosis in arterial thrombosis (17). Conversely, from a functional point of view, the pathophysiological basis for the appearance of miRNA-143-3p, miRNA-125a-5p and miRNA-125b-5p derived from platelets as the proposed major source has not yet been solved. Although platelet activation and aggregation are involved in most of the cardiovascular diseases associated with thrombus formation, the three indicated miRNAs were not recognized as markers in a recent study concerned with acute coronary syndrome (18).

Finally, the contribution of neutrophils in thrombus formation at various sites, including cerebral occlusions, has recently been appreciated (19). A hallmark of neutrophil activation, particularly promoted by platelets in a bidirectional manner to favor thrombosis, is the generation of "neutrophil extracellular traps (NETs)". They represent the extracellular decompensated nuclear DNA-histone network of neutrophils that becomes expelled upon cellular activation and that provides a potent pro-coagulant surface (20). NETs are further characterized by associated proteolytic enzymes as well as by citrullinated histone 3, a protein modification which is required for the generation of NETs (21). Interestingly enough, in AIS patients, NETs were identified in plasma as well as in ischemic stroke thrombi (19,22), indicative for the fact that NETs may serve as a new (prognostic) biomarker for AIS. Yet, further clinical studies need to demonstrate and validate this hypothesis. Since arterial as well as venous thrombi that contain considerable quantities of NETs have been shown to be susceptible towards DNase treatment (23,24), it is proposed that the treatment of ASI patients with a combination of t-PA and DNase instead of standard t-PA alone may yield a more efficient lytic therapy with reduced unwanted bleeding side effects (19,25).

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