SUMMARY OF THE DOCTORAL THESIS

Biomarkers of Astrocyte Dysfunction in the Prognosis of Acute Ischemic Stroke Patients

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Background. Ischemic stroke (IS) is a leading cause of death and a significant contributor to long-term disability worldwide. Despite major advances in acute stroke interventions, including reperfusion therapy with intravenous thrombolysis and mechanical thrombectomy, which have revolutionized patient care, >50% of stroke patients treated in the time window still do not have favorable outcomes. Neuroimaging techniques (CT and MRI) help identify early ischemic changes and assess salvageable brain tissue, collateral circulation, and cerebral hemodynamics, but they do not provide insights into the biological processes underlying stroke. Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles released by nearly all types of cells. These vesicles are found abundantly in various body fluids, can freely pass the blood-brain barrier, reaching the peripheral blood, and carry specific markers reflecting their cells of origin. They also deliver proteins, lipids, and nucleic acids to the target cells, facilitating cell-to-cell communication in both physiological and pathological conditions. These unique qualities make them an ideal source of non-invasive blood-based biomarkers in stroke diagnosis, treatment, and prognosis. Astrocytes are the most abundant and heterogeneous subtype of glial cells in the central nervous system (CNS). As an important component of the neurovascular unit (NVU), they closely interact and communicate with the other NVU cells, releasing EVs. These astrocyte-derived extracellular vesicles (ADEVs) are actively involved in neuroprotection and neurorepair following stroke, however, besides these benefits, they might also have harmful impacts on the CNS.

The main objective of this doctoral thesis was to explore the astrocyte's response to cerebral ischemia in patients with acute ischemic stroke (AIS) by purifying ADEVs and characterizing their cargo in a dynamic manner throughout the first month, focusing on three key proteins: glial fibrillary acidic protein (GFAP), aquaporin-4 (AQP4), and glial-cell line derived neurotrophic factor (GDNF). We also aimed to determine these EV proteins' biomarker potential in assessing stroke severity and outcomes.

General Methodology. We collected blood samples from AIS patients at three intervals: 24 hours, 7 days, and one month after the onset of symptoms to obtain plasma required further for the isolation of EVs using the ExoQuick ULTRA® EV precipitation kit. The obtained EV suspensions were visualized by transmission and scanning electron microscopy, then captured by magnetic streptavidin beads from the Basic Exo-Flow Capture kit, coupled with a mixture of selected biotinylated antibodies targeting the CD9, CD63, and CD81 tetraspanins, to assess the effectiveness of the EV isolation process. A multiplex bead-based assay using the MACSPlex EV IO kit was also conducted to analyze the expression of various surface proteins, including the tetraspanins, on the isolated vesicles. Then, EVs were captured using magnetic beads coupled with the anti-GLAST antibody, which specifically targets a surface marker of astrocytes. These GLAST-positive EVs were considered the ADEV subpopulation. Next, EV suspensions were subjected to lysis to detect and quantify GFAP, AQP4, and GDNF in their cargo by Western blotting. Finally, data from the laboratory experiments were translated into clinical applications, being correlated with different clinical scales: National Institutes of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS), Barthel Index (BI), Activities of Daily Living Scale (ADL), and Instrumental Activities of Daily Living (IADL). Thus, we assessed the potential of the detected EV proteins as non-invasive, blood-based biomarkers in determining stroke severity and outcomes.

Study 1 represents a literature review that aimed to summarize the current knowledge regarding NVU-derived EVs and their possible roles in AIS, outlining each cell type that constitutes this unit. EVs become a newly defined mode of interaction between NVU cells due to their central role in intercellular communication.

Under ischemic conditions, various microRNAs are altered in these EVs, suggesting their applications as possible diagnostic, therapeutic, and prognostic biomarkers in IS.

Study 2 aimed to evaluate and validate a protocol combining precipitation and bead-based flow cytometry to isolate and characterize tetraspanin- and GLAST-positive EVs from plasma samples of AIS patients. Our findings indicated the effectiveness of EV isolation: the obtained vesicles displayed typical size, morphological features, and surface markers for EVs. They were positive for all three tetraspanins, with CD81 revealing the highest expression among them. This suggests that CD81 might be the most potent indicator of EVs secreted by ischemic cells at 24 h post-injury. Besides tetraspanins, these EVs also expressed CD3 and CD42a, suggesting that certain circulating ischemic EVs may derive from T-lymphocytes and platelets, and CD62P, indicating endothelial and platelet activation. Bead-based flow-sort of GLAST-positive EVs enabled the capture of equal amounts of EVs with uniform capture capabilities from each subject, which was essential for further characterization of ADEV suspensions and analysis of differences in their cargo and dynamics post-IS.

Study 3 aimed to clarify aspects of the dynamics of ADEVs released under ischemic conditions, focusing on GFAP, AQP4, and GDNF in their cargo during the first month after stroke. We observed the up-regulation of these ADEV proteins in patients compared to controls, particularly 24 h following stroke. They also demonstrated time-dependent fluctuations through the first month following injury, suggesting that the ischemia-induced pathophysiological changes may be reflected in the periphery. Intravenous thrombolysis had no observable impact on EV cargo. Western blot analyses revealed multiple bands, indicating different splice variants, isoforms, possible dimers, tetramers, variations in glycosylation, or degradation products of the target EV proteins. EV cargo proved to be a new platform for dynamically evaluating the transition from the acute phase of brain injury to repair over the first month following stroke.

Study 4 aimed to explore the clinical applicability of EVs and their cargo, assessing the biomarker potential of the detected EV proteins following stroke. All three target EV proteins correlated with stroke severity, in particular 24 h following symptom onset. EV GFAP and EV AQP4 were associated with outcomes at 7 days, in contrast, EV GDNF correlated with both 7 days and one-month outcomes. These findings highlight the role of EVs in clinical settings and decision-making processes as potential biomarkers for stroke progression and outcome in the acute-subacute phases of the disease.

Originality of the Thesis. EVs transport specific molecular signatures indicating the internal state of their parent cells. This thesis proposes ADEVs as new non-invasive blood-based biomarkers for direct monitoring of CNS signals in vivo, facilitating the collection of longitudinal data on the astrocyte's function and response to injury. Researchers in the field of EVs have primarily focused on stem cell-derived EVs. In recent years, cell-type specific EVs have attracted growing interest as stroke biomarkers due to their unique molecular properties, which allow highly targeted cell-specific communication. Numerous studies detected different EVs in stroke or characterized the cargo of circulating vesicles, but only a few linked the EV content to distinct cell types within the CNS. The majority of these studies were preclinical research, mostly focusing on microRNAs. Explorations on post-stroke EV proteins are still relatively rare. The GFAP, AQP4, and GDNF have already been assessed in various bodily fluids as potential non-invasive markers. However, none of them have been characterized in the cargo of EVs. The present study is the first in this direction. The differential patterns of EV GFAP, AQP4, and GDNF expressions in patients compared to controls emphasize the importance of EV protein profiling in better understanding the role of astrocytes in the pathophysiology of stroke. This also suggests that the ischemia-induced changes at the level of the CNS can be reflected in the circulation of stroke patients. The study of EVs and their cargo provides perspectives for implementing liquid biopsy techniques in the management of AIS patients. The assessed target EV proteins showed promise for potential biomarkers of disease severity and prognosis following the ischemic event. We propose to integrate EV protein profiling into the stroke protocols, besides clinical, laboratory, and imaging investigations, to individualize patient care.