Study Protocol

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1. Title: Investigation of Protein Heterogeneity in Extracellular Vesicles Derived from Different Human Blood Circulatory Regions (DBCR-EV-p)

2. Research Background and Rationale

Extracellular vesicles (EVs) are small, lipid bilayer-enclosed vesicles that are broadly secreted by nearly all cell types into the extracellular microenvironment. They carry biomolecules such as proteins, lipids, mRNA, and miRNA, and play key roles in intercellular communication and disease regulation. Due to their stability and specificity, EVs have become a major focus in the study of disease diagnosis and prognosis[1].

Blood samples are widely used for clinical EV detection due to their accessibility. Over the past five years, one-third of the EV studies included in the EV-TRACK knowledgebase have used blood-derived EVs[2]. EV detection from blood is increasingly applied in the diagnosis and treatment of neurological diseases. Neurons can release EVs that cross the blood-brain barrier (BBB) into the bloodstream, reflecting the pathological changes of the central nervous system (CNS). Therefore, blood-derived EVs are considered potential biomarkers for the development and progression of neurological diseases.

For instance, abnormal aggregation of α-synuclein (α-Syn) is a hallmark of Parkinson's disease. Elevated levels of α-Syn in blood-derived exosomes of Parkinson's disease patients suggest its potential as a diagnostic biomarker[3]. In patients with ischemic stroke, studies have shown that surface antigens on blood-derived EVs from patients with transient ischemic attack (TIA) differ from those in healthy controls. Markers such as CD8, CD2, and CD62P can help distinguish TIA patients from healthy individuals[4].

In addition, blood-derived EVs can cross the BBB to exert effects within the nervous system and are closely associated with neurological prognosis. For example,

in ischemic stroke patients treated intravenously with mesenchymal stem cells (MSCs), circulating EV levels increased fivefold and correlated with improvements in motor function and brain plasticity as observed by MRI[5]. The concentration of EVs in the blood also reflects the pathological state of the brain. Studies have found that EV levels increase during inflammation and are associated with neural injury and disease[6]. Furthermore, since EVs carry surface markers and cargo that reflect their parent cells, identifying the temporal release patterns of neuron-specific EVs may provide new insights into the pathophysiological changes following stroke.

Currently, most blood-derived EV samples in existing studies are collected from peripheral veins, such as the brachial vein. However, there has been no investigation into the heterogeneity of EV contents across different vascular regions such as arteries and deep veins of the head and neck. EVs are rapidly cleared from circulation—studies in mice have shown that 70% of intravenously administered EVs are eliminated from the bloodstream within 2 minutes.[7] Therefore, EVs extracted from blood in the internal jugular vein (which directly drains metabolic products from brain tissue) and from the internal carotid artery (which directly supplies blood to the brain) may more accurately reflect the concentration and content changes of CNS-derived EVs following neurological pathology, making them better sources for diagnostic and prognostic biomarker analysis.

Neurointerventional surgery is a minimally invasive, image-guided medical technique. Using microcatheters and microwires, the procedure enables vascular access for imaging or interventional treatment of cerebrovascular diseases. During surgery, blood from head and neck arteries or veins can be collected. The standard procedure involves: after general anesthesia in the neurointerventional suite, the patient undergoes groin disinfection. A femoral artery puncture is performed using the Seldinger technique, followed by insertion of a 6F or 8F femoral arterial sheath. A 6F or 8F guiding catheter is then advanced over a guidewire through the femoral artery, iliac artery, abdominal aorta, thoracic aorta, aortic arch, and common carotid artery into the internal carotid artery or from the subclavian artery into the vertebral artery. A microcatheter is then guided into the aneurysm for treatment with coiling, stent-assisted

coiling, or flow diverters. After successful embolization, all catheters and sheaths are withdrawn, and hemostasis is achieved with compression.

This project aims to collect blood from the femoral vein, internal jugular vein, and internal carotid artery during neurointerventional surgery under general anesthesia for EV extraction and proteomic differential analysis. The study will characterize the heterogeneity of EVs across different vascular regions in the human body and explore diagnostic and prognostic biomarkers for aneurysmal subarachnoid hemorrhage.

3. Objectives of the Study

1. To reveal the proteomic heterogeneity of extracellular vesicles (EVs) across different vascular regions in the human body
2. To characterize the differences in EV proteomics within cerebral feeding arteries and draining veins between patients with aneurysmal subarachnoid hemorrhage and those without hemorrhage
3. To explore EV-derived protein biomarkers for the diagnosis and prognosis of subarachnoid hemorrhage

4. Study Design

4.1 Study Site and Study Population

This is a prospective, single-center clinical study. Eligible participants will be selected from patients admitted to the Department of Neurovascular Surgery at the First Hospital of Jilin University who are undergoing neurointerventional surgery under general anesthesia for either ruptured or unruptured intracranial aneurysms. During the procedure, blood samples will be collected from the femoral vein, internal jugular vein, and internal carotid artery for subsequent proteomic analysis.

Inclusion Criteria:

1. Aged between 18 and 60 years, regardless of gender;
2. Diagnosed with intracranial aneurysm confirmed by MRA, CTA, or DSA;
3. For patients with aneurysmal subarachnoid hemorrhage, diagnosis must be confirmed by head CT imaging, and blood sample collection must occur within 3 days

of symptom onset;

4. Scheduled to undergo neurointerventional surgery for intracranial aneurysm under general anesthesia, with the procedure starting between 8:00 AM and 12:00 PM;

5. Willing to comply with the study protocol and data collection procedures;

6. Able to understand and sign the informed consent form.

Exclusion Criteria:

1. Modified Rankin Scale (MRS) score ≥ 2 prior to onset in hemorrhagic cases or preoperatively in non-hemorrhagic cases;

2. Presence of other neurological diseases such as Parkinson's disease, Alzheimer's disease, cerebral infarction, brain tumors, or epilepsy;

3. Presence of other systemic diseases such as diabetes, coronary artery disease, cancer, infections, hematological disorders, or severe metabolic diseases that may significantly affect the evaluation of EVs in blood;

4. History of severe hepatic or renal dysfunction (ALT > 3× upper limit of normal; creatinine > 225 μmol/L);

5. Inability to tolerate anesthesia or anticoagulant therapy, or presence of coagulation disorders;

6. History of severe allergic reactions to contrast agents;

7. Pregnant women;

8. Participation in other clinical trials with incomplete follow-up

4.2 Sample Size Determination Method

This is an exploratory study, and there are currently no similar reports in domestic or international literature. As the primary outcome is proteomic differences, potentially involving hundreds of differential results, the sample size has been preliminarily set based on the planned research budget and clinical experience. A total of 60 cases of aneurysmal subarachnoid hemorrhage and 60 cases of unruptured intracranial aneurysms will be enrolled. Proteomic analysis of extracellular vesicles from the first 30 patients will be used to identify intergroup differential markers of interest, which

will inform potential adjustments to the sample size in the subsequent phase of the study.

4.3 Data Collection

Eligible patients will be screened based on inclusion and exclusion criteria. After obtaining informed consent, the following data will be collected: enrollment date, group classification, age, sex, Hunt-Hess grade, Fisher grade, Glasgow Coma Scale (GCS) score, time interval from hemorrhage onset to blood collection, complete blood count, blood biochemistry, perioperative medications, history of hypertension, smoking history, and modified Rankin Scale (mRS) score at discharge (as a prognostic indicator). During neurointerventional surgery under general anesthesia, 5 mL of blood will be collected from each of the femoral vein, internal jugular vein, and internal carotid artery for subsequent proteomic detection and analysis.

Blood Collection Procedure:

1. After general anesthesia takes effect and vital signs are stable, the primary surgeon performs femoral vein puncture and inserts a vascular sheath.
2. Under guidance with a guidewire, a catheter is advanced to the internal jugular vein on the aneurysm side. Through the catheter, 5 mL of blood is collected from the internal jugular vein, and the guiding catheter is then withdrawn.
3. Another 5 mL of blood is collected from the femoral vein through the vascular sheath. After collection, the sheath is removed and the puncture site is compressed for 3–5 minutes. If active bleeding is observed, compression is continued for an additional 3–5 minutes until hemostasis is achieved.
4. The vascular sheath and catheter are thoroughly flushed with heparinized saline.
5. Femoral artery puncture is then performed to insert an arterial sheath. Under guidewire guidance, a catheter is advanced to the internal carotid artery on the aneurysm side. Five milliliters of blood are collected from the internal carotid artery via the guiding catheter, followed by thorough flushing with heparinized saline.
6. A microwire and microcatheter are then delivered through the catheter into the

intracranial vasculature for interventional treatment of the aneurysm.

Blood is collected using EDTA tubes. After collection, each tube should be gently inverted 10 times to prevent coagulation.

4.4 Data Management and Statistical Analysis Plan

Data Management:

A database will be established using electronic spreadsheets.

Statistical Analysis:

Continuous variables will be expressed as mean ± standard deviation (SD), and categorical variables will be presented as absolute numbers and percentages. Statistical analysis will be performed using SPSS version 26.0 (IBM Corp, Armonk, NY). Comparisons between two groups for continuous variables will be conducted using the independent two-tailed Student's t-test. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) will be applied, followed by Newman–Keuls post hoc multiple comparisons test. Differences in categorical variables between groups will be assessed using the chi-square test. A p-value of < 0.05 will be considered statistically significant.

Handling of Missing or Erroneous Data:

For subjects with incomplete observations, the Last Observation Carried Forward (LOCF) method will be applied. If necessary, alternative imputation methods may be used for sensitivity analysis. For erroneous or implausible data, a root cause analysis will be conducted, and data may be excluded if necessary.

4.5 Bias Control

To reduce confounding caused by age and comorbid systemic conditions, patients will be strictly screened according to the inclusion and exclusion criteria, enrolling only those aged 18–60 years without serious systemic diseases other than intracranial aneurysm or subarachnoid hemorrhage. To minimize the impact of time-dependent changes in EV protein composition, only patients whose blood samples are collected within 3 days of hemorrhage onset and whose surgeries are performed between 8:00

AM and 12:00 PM will be included.

To reduce variability in blood lipoprotein levels caused by diet, only patients who have fasted for at least 8 hours before surgery under general anesthesia will be included. Plasma will be prepared within 1 hour of blood collection to minimize the confounding effect of platelet-derived EV release. EVs will be extracted within 12 hours of plasma preparation, and EV suspensions will be snap-frozen in liquid nitrogen and stored at –80°C for subsequent proteomic analysis. This approach aims to reduce changes in EV content due to processing delays.

4.6 Quality Management

The principal investigator is responsible for overall quality control and for clearly defining and executing the responsibilities of all study personnel. Prior to study initiation, all investigators will be trained on the study protocol by the study team. All research staff involved in this study must have valid GCP (Good Clinical Practice) certification and adhere strictly to the clinical trial protocol, using standardized operating procedures to ensure quality control and assurance.

All source data must comply with GCP requirements, and laboratory test results must be accurate and reliable. The principal investigator will conduct regular and systematic reviews of trial-related activities and documentation to assess whether the study is being conducted in accordance with the protocol, standard operating procedures, and applicable regulations, and to ensure that study data are recorded in a timely, truthful, accurate, and complete manner.

4.7 Safety Evaluation

This study involves patients undergoing neurointerventional procedures under general anesthesia. Blood from the internal carotid artery will be collected directly during surgery, posing no additional safety risks. Prior to arterial intervention, femoral vein puncture and sheath placement will be performed to obtain blood samples from the femoral and internal jugular veins.

Femoral vein catheterization is a standard neurointerventional procedure,

commonly used in venous access for treating cerebrovascular malformations or dural sinus diseases. It is also the gold standard for venous blood sampling in the diagnosis of Cushing's syndrome. The complication rate for femoral vein catheterization for cerebral venous sampling is extremely low. Although in theory there may be risks such as infection, excessive bleeding, thromboembolism, or vascular injury, these are rarely encountered in actual practice.

In a clinical study involving 246 patients with Cushing's syndrome undergoing femoral vein catheterization for cerebral venous blood sampling, the only observed complication was mild femoral vein hematoma in a few cases, with no major procedure-related adverse events reported[8].

4.8 Ethical Review and Informed Consent

Ethical Approval:

This study will adhere to the ethical principles outlined in the Declaration of Helsinki and comply with the "Good Clinical Practice for Medical Device Trials" and relevant national regulations. The study protocol will be submitted for approval by the hospital's ethics committee prior to implementation. If amendments to the protocol are required during the course of the study, they must be reviewed and approved by the ethics committee before implementation.

Informed Consent:

Before the clinical trial begins, investigators must provide detailed information about the study to the prospective participants or their legal guardians (authorized representatives). The informed consent form must include the following: the name and contact information of the investigator, the name of the trial site, the title, purpose, methods, and procedures of the study, trial duration, funding sources, potential conflicts of interest, expected benefits and known or foreseeable risks, and potential adverse events.

Participation in the study must be voluntary, and participants have the right to withdraw at any time without discrimination or retaliation, and without affecting their medical care or rights. Participants must be informed that their personal data will be

kept confidential, but may be accessed by the ethics committee, health authorities, or researchers when required according to standard procedures.

In the event of any study-related harm, participants will be eligible for treatment and financial compensation. During the study, participants have the right to access relevant information about themselves and may receive other related compensation or support. The clinical trial may only proceed after the participant or their legal representative has fully understood the study, agreed to participate, signed and dated the informed consent form, and the investigator or their delegate has also signed and dated the form. For each participant, accurate contact details must be recorded. The investigator must also provide their contact information to the participant or their legal representative to ensure that they can be reached promptly in the event of any medical changes. This also facilitates the investigator's ability to monitor clinical progress in real time.

5. References

[1] Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:27066. doi: 10.3402/jev.v4.27066

[2] EV-TRACK Consortium; Van Deun J, Mestdagh P, Agostinis P, Akay Ö, Anand S, et al.EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nat Methods. 2017 Feb 28;14(3):228-232. doi: 10.1038/nmeth.4185.

[3] Shi, M., Liu, C., Cook, T. J., et al. (2014). Plasma exosomal α-synuclein is likely CNS-derived and increased in Parkinson's disease. Acta Neuropathologica, 128(5), 639-650. doi:10.1007/s00401-014-1314-y

[4] Burrello J, Bianco G, Burrello A, Manno C, Maulucci F, Pileggi M, NannoniS, Michel P, Bolis S, Melli G, et al. Extracellular vesicle surface markers as a diagnostic tool in transient ischemic attacks. Stroke. 2021;52:3335–3347. doi: 10.1161/STROKEAHA.120.033170

[5] Bang OY, Kim EH, Cho YH, Oh MJ, Chung JW, Chang WH, Kim YH, Yang SW,

Chopp M. Circulating Extracellular Vesicles in Stroke Patients Treated With Mesenchymal Stem Cells: A Biomarker Analysis of a Randomized Trial. Stroke. 2022 Jul;53(7):2276-2286. doi:10.1161/STROKEAHA.121.036545

[6] Marostica G, Gelibter S, Gironi M, Nigro A, Furlan R. Extracellular Vesicles in Neuroinflammation. Front Cell Dev Biol. 2021 Jan 21;8:623039. doi: 10.3389/fcell.2020.623039

[7] Kang M, Jordan V, Blenkiron C, Chamley LW. Biodistribution of extracellular vesicles following administration into animals: A systematic review. J Extracell Vesicles. 2021 Jun;10(8):e12085. doi: 10.1002/jev2.12085.

[9] Oldfield EH, Doppman JL, Nieman LK, Chrousos GP, Miller DL, Katz DA, Cutler GB Jr, Loriaux DL. Petrosal sinus sampling with and without corticotropin-releasing hormone for the differential diagnosis of Cushing's syndrome. N Engl J Med. 1991 Sep 26;325(13):897-905. doi: 10.1056/NEJM199109263251301