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Study Protocol

1. Background

Research on the relationship between biomarkers in human cerebrospinal fluid (CSF) and aging is a current hotspot in the field of neuroscience. Analyzing specific proteins and other molecules in CSF can provide important information for the early diagnosis and pathological progression of neurodegenerative diseases. Common biomarkers in CSF include amyloid-beta (Aβ42) and phosphorylated tau protein (p-tau181)1. The concentration changes of these markers have significant diagnostic value in Alzheimer's disease (AD) and other neurodegenerative diseases. A decrease in Aβ42 levels and an increase in p-tau181 levels are closely associated with the pathological features of AD2. Aging is a major risk factor for neurodegenerative diseases, and studies have found that the levels of Aβ and tau proteins in CSF undergo significant changes with increasing age. These changes are particularly evident in neurodegenerative diseases such as Alzheimer's disease and Lewy body dementia3. Research shows that monitoring biomarkers in CSF can aid in the early identification of these diseases, thereby facilitating early intervention and treatment4. Modern studies utilizing high-throughput proteomics and other advanced biotechnologies have revealed many new potential biomarkers in CSF. For example, mass spectrometry can detect protein changes related to neuroinflammation and blood-brain barrier function, which are crucial for understanding the neurodegenerative pathological mechanisms during aging5.

In-depth research into CSF biomarkers not only aids in understanding the neurodegenerative changes during aging but also promotes the development of early diagnostic techniques, improving the management and treatment of neurological diseases in the elderly population. Future research will further explore the mechanisms and applications of these biomarkers, aiming to achieve broader and more precise diagnosis in clinical practice.

References:

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2. Mattsson-Carlgren N, Grinberg LT, Boxer A, et al. Cerebrospinal Fluid Biomarkers in Autopsy-Confirmed Alzheimer Disease and Frontotemporal Lobar Degeneration. Neurology. 2022;98(11):e1137-e1150. doi:10.1212/WNL.0000000000200040

3. O'Leary K. A timeline of biomarker changes before Alzheimer's disease. Nat Med. Published online March 15, 2024. doi:10.1038/d41591-024-00018-0

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5. Krance SH, Cogo-Moreira H, Rabin JS, Black SE, Swardfager W; Alzheimer's Disease Neuroimaging Initiative. Reciprocal Predictive Relationships between Amyloid and Tau Biomarkers in Alzheimer's Disease Progression: An Empirical Model. J Neurosci. 2019;39(37):7428-7437. doi:10.1523/JNEUROSCI.1056-19.2019

2. Objective

Analyzing CSF samples from different age groups to identify biomarkers that undergo significant changes during the aging process, such as amyloid-beta (Aβ42), tau protein (including its phosphorylated form, p-tau181), and other proteins related to neuroinflammation and blood-brain barrier function.

Establishing a predictive model through the analysis of CSF protein levels and classification across different age stages, to enable the early diagnosis of neurodegenerative diseases (e.g., Alzheimer's disease). This could allow for the detection of diseases before clinical symptoms appear, providing the possibility for early intervention and treatment.

Investigating the dynamic changes of CSF biomarkers at different stages of disease to understand their roles in disease progression, including changes in the early, middle, and late stages. This will help to better understand the pathological process and identify potential

therapeutic windows.

Creating a database of CSF biomarkers that encompasses different age groups, disease states, and treatment responses. This database will provide valuable data resources for future research, facilitating the scientific community's understanding of neurodegenerative diseases and the development of new therapies.

Through the achievement of these objectives, this study aims to advance the development of CSF biomarkers in neuroscience research and clinical applications, improving the diagnosis, management, and treatment of aging and neurodegenerative diseases.

3. Method

Prospective Collection of Cerebrospinal Fluid from Patients Aged 0-90 Years Without Neurological Diseases:

Study Duration: 1 year, starting from the date of ethical approval and ending 1 calendar year after the date of ethical approval.

Inclusion Criteria:

Patients aged 0-90 years.

Patients admitted for surgery under spinal anesthesia due to non-neurological conditions such as lower limb fractures, hemorrhoids, etc.

Pediatric patients requiring surgery for tethered cord syndrome.

Exclusion Criteria: Patients with neurological diseases, including but not limited to encephalitis, Parkinson's disease, Alzheimer's disease, hydrocephalus, brain tumors, and psychiatric disorders.

Withdrawal Criteria: Participants may voluntarily withdraw from the study at any time for any reason.

Specific Research Protocol:

Data Collection: Clinical data will be collected, including the patient's name, gender, age, and primary diagnosis. This information will be sourced from the hospital admission case system.

Cerebrospinal Fluid (CSF) Collection:

During spinal anesthesia, CSF is withdrawn to confirm the correct placement in the subarachnoid space before administering the anesthetic, with approximately 1-2 mL of CSF retained.

During surgery for tethered cord syndrome, 2 mL of CSF is collected before opening the dura mater.

The collected CSF is placed in a 0°C insulated container for storage and transportation, then centrifuged in the laboratory and stored at -80°C until further analysis.

After the collection is complete, multi-omics analyses, including proteomics and metabolomics, will be conducted.

Proteomics Analysis:

Sample Preparation:

100 μL of CSF is mixed with high-abundance protein depletion resin to enhance proteome depth, incubated at room temperature for 20 minutes, and the eluate is collected.

The sample is concentrated using a 3k molecular weight cutoff protein concentrator at 12,000 g for 30 minutes and exchanged with lysis buffer (8M urea) for protein denaturation.

After centrifugation at 12,000 g, less than 50 μL of solution remains in the tube.

The concentrated sample is reduced with 10 mM tris(2-carboxyethyl)phosphine hydrochloride at 32°C for 40 minutes, followed by alkylation with 40 mM iodoacetamide at room temperature in the dark for 30 minutes.

CSF samples are digested in two steps using 2.5 μg of endoproteinase and 2.5 μg of trypsin for 12 hours.

The peptides are acidified with trifluoroacetic acid, desalted using a 2 mg liquid-phase ion exchange column, and the eluate is dried using a vacuum concentrator.

Each batch is labeled with 56 μg of TMT16plex reagent for peptides from each sample. After 1-hour incubation at room temperature, TMT labeling is quenched with

hydroxylamine, and 16 samples from a batch are pooled and fractionated using a C18 column.

Fractions are separated into 96 fractions with a gradient from 5% to 35% acetonitrile in 10 mM ammonia water at a flow rate of 1 mL/min. The first to seventh, 37th, and 67th fractions are pooled, and the remaining fractions are evenly pooled and vacuum dried.

The dried peptide powder is reconstituted in 2% acetonitrile/0.1% formic acid and analyzed by LC-MS/MS.

Data Analysis:

MS raw data is searched against the human protein fasta file downloaded from UniProtKB14, which contains 20,394 protein entries.

The parent ion mass tolerance is set to 10 ppm, and the fragment ion mass tolerance is set to 0.02 Da.

Subsequent statistical analysis is performed by comparing the abundance ratios of samples within the same batch to the combined sample.

Bioinformatics software is used to analyze the mass spectrometry data, including protein identification, quantification, and differential analysis.

4. sample capacity

Participants will be divided into 9 groups based on age, with each group covering a 10-year range (1-10 years, 11-20 years, etc.), totaling 9 groups. Each group will include 10-15 participants, with a planned total enrollment of 100-150 cases.

5. Data management and confidentiality

Data Entry and Modification:

Data entry and management will be handled by Zhu Zhoule and Ying Yuqi. To ensure accuracy, two data administrators will independently enter and cross-check the data.

Database Locking:

Once the database has been confirmed to be accurate, it will be locked by the principal investigator and statistical analysts. All records related to participant identities will be kept confidential and will not be disclosed outside the scope allowed by relevant laws and/or

regulations.

6. informed consent

Before the clinical study begins, the researchers must provide detailed information to the participants or their legal guardians, including the nature of the study, its purpose, potential benefits, and risks. This ensures that participants or their legal guardians fully understand the clinical study. The clinical study can only commence after the informed consent form has been signed.

Each patient must provide detailed contact information, including their address and phone number. Additionally, the researcher will sign the informed consent form with the patient before the surgery and provide the patient with their contact information so that the patient can reach the researcher at any time.

7. Adverse Event Reporting

This study involves the collection of 1-2 mL of cerebrospinal fluid (CSF) under spinal anesthesia. A very small number of patients may experience headaches or discomfort after the procedure, which can be alleviated by increasing fluid intake.

General Adverse Events: Timely measures will be taken to address any adverse events, and they will be recorded in the case report form.

Serious Adverse Events (SAE): Timely measures will be taken to address any SAE. The event will be recorded in the case report form, and the researcher will decide whether to stop or reduce treatment. The SAE must be reported immediately to the Ethics Committee, the drug clinical trial institution, and the sponsor. Additionally, within 24 hours, it must be reported to the National and Provincial Food and Drug Administration. The SAE must also be reported through the "Internal Adverse Event and Near-Miss No-Fault Reporting System." For the detailed process, see the diagram below.