Annex 3.

Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine

Research projects involving human subjects

(Applicable to prospective studies)

Study Title:	Predictive Role of Early Blood KLK10 and Other Neuroinflammatory Molecules on Clinical Outcomes of Acute Ischemic Stroke
Protocol Number:	2024092
Principal Investigator:	Longxuan Li
Department:	Neurology
Study Period:	April 2024 - March 2025

Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine

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1. Research Abstract

1.1 Abstract

Study Name: Predictive Role of Early Blood KLK10 and Other Neuroinflammatory Molecules on Clinical Outcomes of Acute Ischemic Stroke

Research Introduction: This study aims to investigate the dynamic changes and differential expression characteristics of neuroinflammatory molecules such as kallikrein-related peptidase 10 (KLK10), soluble triggering receptor expressed on myeloid cells 2 (sTREM2), and glial fibrillary acidic protein (GFAP) in the plasma of patients with first-ever anterior circulation acute ischemic stroke (AIS). It will analyze the correlation between these molecules and the severity of neurological deficits, infarct volume, brain edema, hemorrhagic transformation, progressive stroke, and clinical outcomes at three months. The goal is to assess the predictive value of these molecules for AIS prognosis, providing guidance for early treatment and new molecular targets for prevention and treatment.

Study Objectives :

1. To study the correlation between early plasma changes in neuroinflammatory molecules like KLK10 and clinical outcomes at three months post-AIS.
2. To investigate the relationship between early plasma changes in these molecules and hemorrhagic transformation post-AIS.
3. To explore the correlation between early plasma changes in these molecules and the occurrence of progressive stroke post-AIS.

Study subjects: 182 patients with first-ever anterior circulation AIS, regardless of gender and age, along with 91 age- and gender-matched healthy individuals as controls.

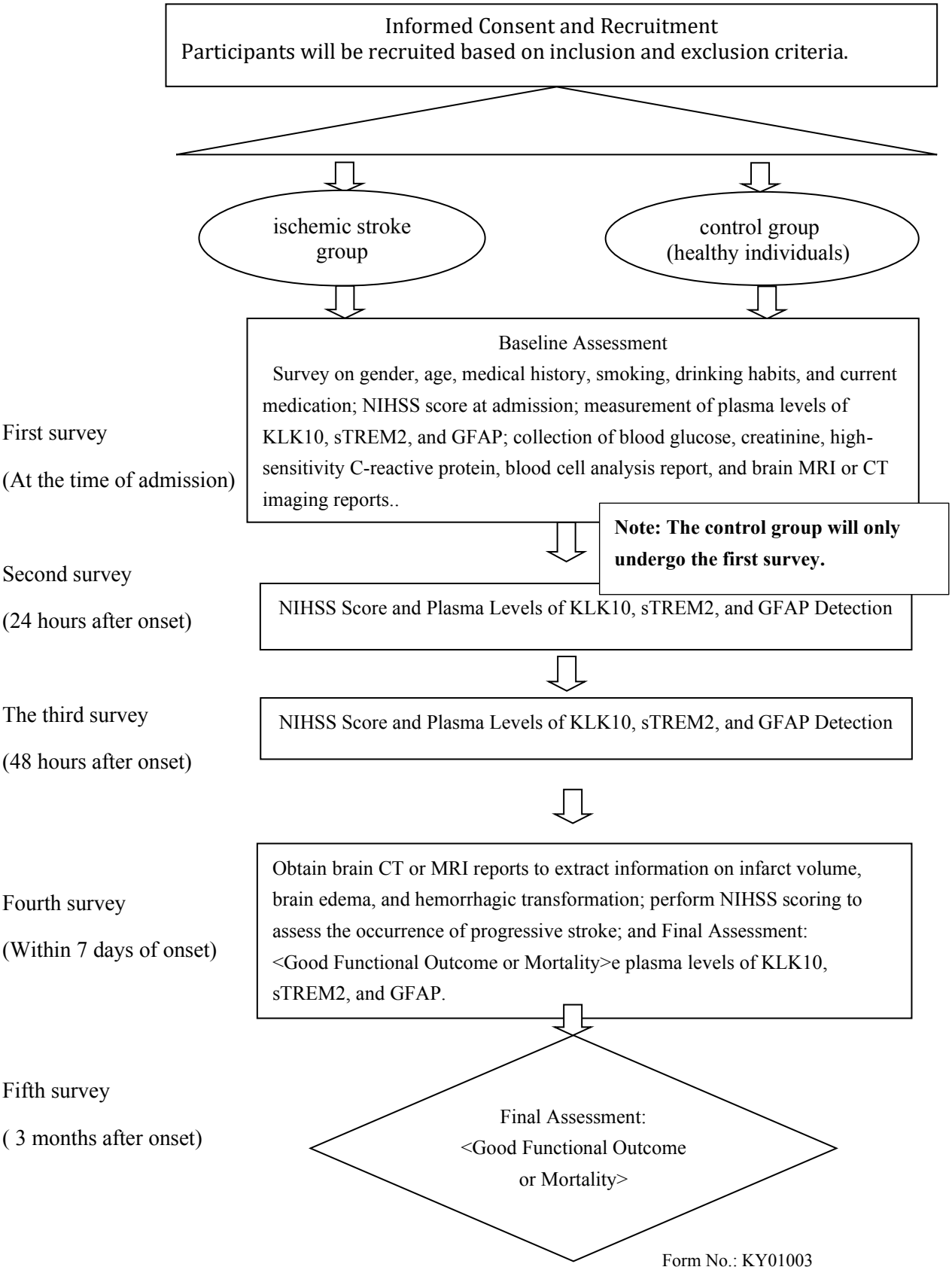
Research Site: Ruijin Hospital North Campus

Study Interventions : Non-interventional studies

Study duration: April 2024 - March 2025

Participant Duration: 3 months

1.2 Technology Roadmap



2. Study background

2.1 Research Significance

Stroke is the leading cause of death and disability in China. Intravenous thrombolysis or thrombectomy are currently the only approved early treatments for AIS (acute ischemic stroke). However, due to the limited time window, the number of patients benefiting from these treatments is restricted, leaving many patients with cerebral infarction. Studies have shown that neuroinflammatory molecules rapidly activate post-infarction and are involved in the pathogenesis of cerebral infarction. Moderate inflammation helps clear necrotic tissue and plays a crucial role in tissue repair and remodeling, while excessive inflammation can exacerbate ischemic neuronal damage and impair recovery (*Lancet Neurol, 2019, 18(11): 1058-1066*). Inflammatory markers in the blood rise quickly after 2 hours of stroke onset and continue to increase within 24 hours (*Stroke.2022;53(7):2249-2259*), making early detection of blood neuroinflammatory markers significant for predicting clinical outcomes in AIS. This study aims to dynamically measure changes in a set of neuroinflammatory molecules in the plasma, including KLK10, and analyze their correlation with the severity of acute neurological deficits, infarct volume, brain edema, hemorrhagic transformation, progressive stroke, and clinical outcomes at three months. Given the time-sensitive and challenging nature of AIS management, confirming that a set of neuroinflammatory molecules like KLK10 can accurately predict post-stroke clinical outcomes would have significant scientific value for guiding early treatment of AIS and could provide new molecular targets for stroke prevention and treatment.

2.2 Research Background

Microglia, the primary immune cells in the brain, are among the first cells to activate after AIS, playing a critical role in accelerating inflammation, increasing or reducing neuronal apoptosis, and regulating the post-stroke inflammatory microenvironment, thus affecting stroke prognosis (*J Neuroinflammation. 2022;19(1):88*). KLK10, also known as normal epithelial cell-specific molecule-1, is one of the 15 secreted serine proteases in the kallikrein family. Previous studies have shown that KLK10 is expressed in endothelial cells, where it participates in regulating endothelial inflammation and atherosclerosis (*Elife. 2022;11:e72579*). Our recent systematic observations of KLK10's spatial-temporal expression and cellular distribution in the brain after ischemia revealed that this molecule is expressed not only in cerebral endothelial cells but also in microglia and astrocytes. While it did not significantly increase in

the ischemic penumbra in the early stages (2-4 days post-ischemia), it was highly expressed in the infarct core area at 2 days post-ischemia and significantly co-labeled with microglia. The specific high expression of KLK10 in microglia in specific brain regions post-AIS suggests that it may be involved in early pathological damage after cerebral ischemia. Given that the permeability of the blood-brain barrier (BBB) changes within 6 hours of stroke onset (*Front Neurol. 2020;11:594672*), and since KLK10 is a secreted molecule, it is likely that its expression can be detected in the blood of AIS patients. Whether plasma KLK10 levels can reflect changes in neuroinflammatory damage in AIS patients and serve as a specific biomarker for predicting AIS prognosis remains unclear and warrants further investigation.

TREM2 is an innate immune receptor in the brain, consisting of an extracellular immunoglobulin-like domain, a transmembrane domain, and a cytoplasmic portion. Post-stroke, TREM2 is activated by nucleotides, lipid mediators, and other key factors released by apoptotic neurons, cells/myelin debris, playing an important role in regulating neuronal damage (Front Immunol, 2019, 10: 1668). Literature shows that TREM2 molecules are mainly expressed in microglia and can reflect their activation status. Elevated plasma sTREM2 levels are closely associated with increased risk of death and cardiovascular events one year after AIS onset (*J Neuroinflammation. 2022;19(1):88*).

Astrocytes, which also play a role in regulating brain inflammation, are the main glial cells in the central nervous system and are widely distributed throughout the brain. In their resting state, astrocytes provide structural and functional support to neurons, regulate synaptic activity by releasing gliotransmitters, and participate in synapse formation and remodeling (*Neurochem Int. 2023;162:105456. Neuroscience, 2019, 396:73-78*). After cerebral ischemia, astrocytes become activated and proliferate, limiting immune cell infiltration, repairing damaged BBB, and restoring stability in the brain's internal environment (*Front Immunol, 2020, 11:1024. Neurosci Lett, 2019; 689: 45-55*). Our studies and others have shown that astrocytes are extensively lost in the infarct core after cerebral ischemia, peaking at 48-98 hours post-stroke. The GFAP expressed by astrocytes can be released into peripheral blood, and its levels in the blood can reflect cell necrosis and BBB disruption *(Transl Stroke Res. 2010;1(4):246-51)* and can serve as molecular markers for distinguishing ischemic and hemorrhagic strokes (*Diagnostics (Basel). 2023 Aug 25;13(17):2757*).

The pathological damage mechanism of stroke is highly complex. Single neuroinflammatory molecules in plasma, whether sTREM2 or GFAP, are insufficient for predicting AIS prognosis. However, measuring a set of neuroinflammatory molecule levels may more accurately reflect the degree of neuropathological damage in AIS. Therefore, we propose that "early changes in plasma levels of KLK10, sTREM2, and GFAP, among other neuroinflammatory molecules, can effectively predict the clinical prognosis of AIS."

2.3 Expected Outcomes

To reveal whether early plasma levels of KLK10 and other neuroinflammatory molecules can serve as significant molecular markers for predicting the prognosis of AIS patients.

2.4 Risk/Benefit Assessment

2.4.1 Known Potential Risks

This study requires collecting 10ml of peripheral blood from AIS patients, with a total of three collections, and from healthy individuals, with a total of one collection. There is no significant potential risk to patients or healthy individuals, with the rare occurrence of fainting after blood draw.

2.4.2 Known Potential Benefits

The study does not provide significant benefits to patients, but the new findings from this study may help guide future treatment of stroke.

2.4.2 Potential Risk/Benefit Assessment

Given the rare occurrence of fainting in some patients after peripheral blood collection, symptomatic treatment will be administered. The success of this project has significant scientific value for guiding early treatment of AIS and providing new molecular targets for stroke prevention and treatment, potentially bringing important economic value.

3. Principal Investigator Information

3.1 Principal Investigator's Name, Qualifications, and Contact Information

Longxuan Li, Professor, Chief Physician

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Email: llx12472@rjh.com.cn

3.2 Main Participants

Serial number	Name	gender	age	job title	Specialty	Whether GCP Training	Roles in the research (eg. PI , sub-I, CRC)
1	Longxuan Li	male	51	Chief Physician	Neurology	yes	PI
2	Bean YIn	male	38	Deputy Chief Physician	Neurology	yes	sub-I
3	Wei Jin	male	38	Indications Physician	Neurology	yes	sub-I
4	Yi Zhang	male	33	Indications Physician	Neurology	yes	CRC
5	Pingchen Zhang	female	29	Hospitalization Physician	Neurology	yes	CRC

4. Research Objectives

1) To determine whether early KLK10 and a group of neuroinflammatory molecules in plasma can serve as molecular markers for predicting clinical prognosis in AIS (Acute Ischemic Stroke).
2) To determine whether early KLK10 and a group of neuroinflammatory molecules in blood can serve as molecular markers for predicting hemorrhagic transformation in AIS.

5. Study Design

5.1 Overall Design

A total of 182 patients with first-ever anterior circulation AIS (Acute Ischemic Stroke) hospitalized in the Neurology Department at the Northern Campus of our hospital will be selected as the observation group, and 91 healthy individuals undergoing physical examination will be selected as the control group. For AIS patients, 10 ml of elbow venous blood will be collected at admission and then at 24 hours and 7 days after stroke onset. For healthy individuals, 10 ml of fasting elbow venous blood will

be collected once. The dynamic changes and differential expression characteristics of a group of neuroinflammatory molecules, including KLK10, sTREM2, and GFAP in plasma will be observed. The early changes (at admission and 24 hours after onset) of these neuroinflammatory molecules in plasma will be compared and analyzed in relation to the severity of acute neurological deficits (NIHSS score), infarct volume, brain edema, hemorrhagic transformation, occurrence of progressive stroke, and clinical prognosis at 3 months (mRS score), to evaluate the predictive value of these molecules for clinical outcomes after AIS.

5.2 Defining study endpoints

The study is completed when patients have completed blood sample collection according to the study protocol and follow-up at 3 months after stroke onset. For healthy controls, the study is completed after the initial blood sampling and baseline investigation.

5.3 Determining sample size

Assuming a 40% probability of poor prognosis at 3 months after AIS, with an OR of 2.0 for high KLK10 expression and a correlation coefficient of 0.5 between KLK10 and other influencing factors, a significance level of 0.05, and a test power of 0.8, the required sample size is calculated using PASS11 software's Logistic Regression module. A total of 182 AIS cases are needed. Additionally, 91 age- and gender-matched individuals will be selected as healthy controls in a 2:1 ratio.

6. Research subjects

6.1 Inclusion Criteria

Inclusion criteria for AIS patients:

- Selection of 182 patients with first-ever acute ischemic stroke (AIS) who are hospitalized at our hospital, aged ≥18 years, with no restriction on gender;
- Patients must be admitted within 24 hours of the onset of AIS;
- Diagnosis must be confirmed by cranial magnetic resonance imaging (MRI) or computed tomography (CT);
- All enrolled patients must provide written informed consent.

Inclusion criteria for healthy controls:

- Age and gender matched;
- No organic diseases;
- Written informed consent must be signed

6.2 Exclusion criteria

- Intracranial hemorrhage;
- Pregnancy;
- Stroke with unknown onset time;
- Malignant tumors;
- Hematologic disorders;
- Severe liver or kidney dysfunction;
- Recent myocardial infarction (less than 3 months);
- Ongoing anti-inflammatory drug treatment

6.3 Recruitment of research subjects

- Source of subjects: AIS patients will be recruited from inpatients in the Department of Neurology at the Northern Campus; healthy controls will be selected from outpatient health check-up populations who have undergone routine blood tests, biochemical tests, cranial CT, or MRI
- Recruitment site: Ruijin Hospital Northern Campus

6.4 Method of allocating research subjects

not applicable

7. Study Intervention

7.1 Administration of Study Intervention

not applicable

7.1.1 Description of study intervention

not applicable

7.1.2 Dosage and administration method

not applicable

7.1.3 Establishment, Storage, Unblinding Method, and Emergency Unblinding Procedure for the Trial Drug Code

not applicable

7.1.4 Items and frequency of clinical and laboratory examinations to be conducted

1) Collect reports of complete blood count and biochemical tests upon admission: including neutrophil and lymphocyte counts, blood glucose, creatinine, and high-sensitivity C-reactive protein levels.
2) Collect blood pressure information upon admission.
3) Collect reports of cranial MRI or CT scans upon admission, and record the Alberta Stroke Program Early CT Score (ASPECTS) (0-10 points).
4) Measure levels of neuroinflammatory markers KLK10, sTREM2, and GFAP in plasma at admission, and at 24 hours and 7 days after stroke
5) Assess the degree of neurological impairment using the National Institutes of Health Stroke Scale (NIHSS) at admission, and at 24 hours, 4 days, 7 days, and discharge after stroke.
6) Collect information on infarct volume within 7 days after stroke, measured using diffusion-weighted imaging (DWI) (J Neuroinflammation. 2020 Apr 7;17(1):107).
7) Collect information on brain edema grading within 7 days after stroke, measured using non-contrast cranial CT scans. The severity of brain edema in AIS patients is assessed with a score of 0-3 based on our established method (Clin Neurol Neurosurg. 2022 Nov 2;223:107507).
8) Collect information on hemorrhagic transformation within 7 days after stroke, measured using non-contrast cranial CT scans.
9) Record the Modified Rankin Scale (mRS) score at 3 months after stroke.

7.2 Preparation/Handling/Storage/Responsibility

not applicable

7.3 Measures to reduce bias:

not applicable

7.4 Follow-up and Compliance

3 months mRS Score: 0-2 indicates a good outcome, 4-6 indicates a poor outcome
Grading Description:

- 0: No symptoms at all

- 1: No significant disability despite symptoms; able to carry out all usual duties and activities
- 2: Slight disability; unable to carry out all previous activities but able to look after own affairs without assistance
- 3: Moderate disability; requiring some help but able to walk without assistance
- 4: Severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
- 5: Very severe disability; bedridden, incontinent, and requiring constant nursing care and attention
- 6: Death

7.5 Research Intervention Commitment

not applicable

7.6 Research Plan

Visit	Screening period	Observation period				Follow-up period
	1 day after onset	On admission	24 hours after onset	24 hours after onset	7 days after onset	3 months after onset
Informed consent	×					
Inclusion/Exclusion Criteria	×					
Basic information collection	×					
Peripheral venous blood collection		×	×	×	×	
NIHSS score		×	×	×	×	
blood sugar		×				
KLK10, sTREM2 and GFAP detection		×	×	×	×	
MRI					×	
CT	×				×	
mRS score						×

8. Discontinuation of study intervention and discontinuation/withdrawal of study subjects

8.1 Discontinuation of Study Intervention

not applicable

8.2 Discontinuation/Withdrawal of Study Subjects

not applicable

8.3 Loss to follow-up

To reduce loss of follow-up and missing data, patients who were not followed up on time were contacted by telephone.

9. Study endpoint evaluation

9.1 Primary Endpoint Evaluation

The study examines the association between plasma levels of KLK10, sTREM2, and GFAP with favorable functional outcomes at 3 months after stroke onset.

9.2 Secondary Endpoint Evaluation

1) Investigate the correlation between plasma KLK10, sTREM2, and GFAP levels and mortality at 3 months after stroke onset.
2) Examine the correlation between plasma KLK10, sTREM2, and GFAP levels and hemorrhagic transformation after infarction, based on brain CT scans to evaluate hemorrhagic transformation of cerebral infarction within 7 days after stroke onset.
3) Explore the association between plasma KLK10, sTREM2, and GFAP levels and the occurrence of progressive stroke, with neurological deficits assessed using the NIHSS score. Progressive stroke is diagnosed if the NIHSS score increases by 2 points within 7 days after stroke onset.

9.2 Safety and other evaluations

not applicable

9.3 Adverse Events and Serious Adverse Events

not applicable

10. Statistical Analysis

10.1 General Methods

For descriptive statistical analysis, measurement data conforming to a normal distribution are expressed as mean ±standard deviation; measurement data not conforming to a normal distribution are expressed as median (interquartile range); count data are expressed as rates or proportions. For comparing two groups of measurement data with normal distribution and equal variance, an independent two-sample t-test is used; for comparing two groups of measurement data not conforming to normal distribution and equal variance, a non-parametric Mann-Whitney U test is used. For count data in contingency tables, Pearson's chi-square test is used if conditions are met; otherwise, Pearson's chi-square test with continuity correction or Fisher's exact test is applied. All tests are two-tailed, with $P<0.05$ considered statistically significant.

10.2 Primary Endpoint Analysis

Investigate the association between plasma KLK10, sTREM2, and GFAP levels and functional prognosis at 3 months: Univariate and multivariate logistic regression models are used to evaluate independent predictors, and odds ratios (OR) with 95% confidence intervals (CI) are calculated. ROC curves are plotted and the area under the curve (AUC) is calculated.

10.3 Analysis of Secondary Study Endpoints

1) The correlation between plasma KLK10, sTREM2, and GFAP levels and hemorrhagic transformation and mortality at 3 months was investigated: univariate and multivariate logistic regression models were used to evaluate independent predictors, and ORs and 95% CIs were calculated. ROC curves were drawn and AUCs were calculated.
2) The association between plasma KLK10, sTREM2 and GFAP levels and the occurrence of progressive stroke was studied: univariate and multivariate logistic regression models were used to evaluate independent predictive factors , calculate OR and 95%CI. ROC curves were drawn and AUC was calculated.

10.4 Security Analysis

not applicable

10.5 Baseline Descriptive Analysis

Descriptive statistics are used to compare demographic characteristics and laboratory indicators at baseline between groups. Continuous variables such as age, blood pressure, blood glucose levels, infarct volume, ASPECTS score, neurological function (NIHSS) score, and levels of neuroinflammatory molecules (KLK10, sTREM2, and GFAP) are expressed as mean (standard deviation) or median (interquartile range). Categorical variables such as the presence of hypertension, diabetes, hyperlipidemia, history of stroke, and occurrence of malignant brain edema are expressed as n (percentage). Fisher's exact test is used to assess differences in categorical variables, and the Kruskal-Wallis Rank Sum test is used to detect differences in continuous variables..

10.6 Subgroup Analysis

not applicable

11. Supporting documents and notes

11.1 Informed Consent Process

Before the study begins, the researcher must obtain approval from the Ruijin Hospital Ethics Committee. An informed consent form should be provided to the research subjects. The procedure for obtaining informed consent should be described, including how the consent is obtained, the environment during the signing, and issues such as signing on behalf of the subject by a guardian. Informed consent should be completed before the research subject or their legal guardian agrees to participate in the study and should continue throughout the study. The informed consent form, approved by the ethics committee, should be read by the research subject. The researcher will explain the study process, answer any questions from the subjects, and inform them of potential risks and their rights. Subjects may discuss participation with their family or guardian before agreeing. The researcher must inform the subjects that participation is voluntary and that they may withdraw from the study at any time. A copy of the informed consent form may be provided to the subjects for their records. The rights and welfare of the subjects will be protected, and it will be emphasized that their quality of medical care will not be affected if they refuse to participate.

11.2 Privacy Protection

Electronic files should be password protected and stored on a computer, while paper files should be kept in a locked cabinet. All data is accessible only to the researchers, and no research information can be disclosed to unauthorized third parties without approval.

11.3 Collection and use of specimens and data

Sample collection :

1) Blood collection and separation method: After the subjects sign the informed consent form, 10ml of peripheral elbow vein blood is collected using EDTA tubes at the time of admission and at follow-up points. The sample should be gently inverted 5-10 times immediately after collection to ensure thorough mixing and reduce the risk of hemolysis. Let it stand at room temperature for 30 minutes. Centrifuge at 2500 rpm for 15 minutes in a high-speed centrifuge. The supernatant is collected, and the serum is stored at -80℃ for subsequent analysis.
2) Basic information and labeling rules for sample submission: For samples submitted for testing KLK10, sTREM2, and GFAP levels in blood from AIS patients and healthy controls, basic information such as the patient's name, age, and date of submission must be provided. The sample numbering principle is: the subject's initials (e.g., Zhang San Si, ZSS), research center number, subject number (e.g., 0001), and follow-up number (e.g., a for admission, b for 24h after onset, and so on).
3) Sample testing: ELISA is used to detect plasma levels of KLK10, sTREM2, and GFAP, following the specific methods in the kit instructions.

After the study ends, the remaining biological samples will be directly destroyed. Imaging data and other data will be stored in electronic files, which should be password protected and stored on a computer. Paper files should be kept in a locked cabinet, and all data is accessible only to the researchers. No research information can be disclosed to unauthorized third parties without approval. All study data and original documents will generally be retained permanently, with permission required before destruction.

11.4 Quality Control and Quality Assurance

All personnel involved in the study should establish a quality assurance system, perform their respective duties, and strictly follow the clinical trial protocol and corresponding standard operating procedures to ensure the implementation of the quality control and quality assurance system for the study. The study will adhere to the relevant SOPs.

Quality control during the study: The personnel assessing the study site should evaluate the basic conditions for participating in the study to ensure they meet protocol requirements. Before initiation, the main investigators at the study site should thoroughly train the study staff on the protocol, ensuring a clear understanding of inclusion and exclusion criteria and the specific content of various indicators. During the study, staff should carefully execute the enrollment and follow-up procedures as required by the protocol, and record data accurately, timely, completely, and in a standardized manner. Quality control personnel will conduct quality checks on the trial process and corresponding original records. After the trial, the study site will organize the relevant project files, which will be checked by quality control personnel before archiving.

Quality control during sample testing: The testing laboratory should have a strict quality assurance system and strictly follow domestic and international technical guidelines, laboratory standard operating procedures, and quality control procedures. This includes, but is not limited to, personnel training and authorization, sample management, standard management, equipment verification, calibration and maintenance, method validation, sample testing, and data verification.

Quality control of data entry: Level 1 quality control: The information automation judgment system on the user side of EDC will initially check the logic and accuracy of the input information, and perform routine logic checks when entering the software (for example, age cannot be filled in as 200, etc.), reminding the data entry personnel to implement corrections before entering the data. Level 2 quality control: The sponsor will send verification personnel to conduct consistency verification of on-site original data and EDC data from time to time. Level 3 quality control: Quality control is carried out by professionals, mainly including the investigation and correction of routine errors in risk assessments such as medicine and epidemiology, to avoid errors in assessment reports. Questionnaires with 1% positive assessment results and 0.5% negative assessment results will be reviewed and re-evaluated to monitor the quality of the assessment.

11.5 Data Processing and Record Keeping

11.5.1 Data Collection and Management

1) Data entry and management: managed by a dedicated person, research staff should ensure the completeness, authenticity and accuracy of the trial data. This study uses the electronic case reporting system (EDC) to collect data from included subjects, and all data should be derived from original records. The original records of this study, including complete examination reports for each follow-up, and clinical diagnosis and treatment information related to stroke, are stored in the research center information system.

2) Clinical data will be stored in a database, which should be password protected and a logical proofreading procedure should be established when the database is created.

11.5.2 Research Data Retention

All research data and original documents will generally be retained permanently and permission should be obtained before they are destroyed.

11.6 Publication and Data Sharing Agreement

not applicable

11.7 Conflict of Interest Statement

not applicable