A Single-Arm, Open-Label, Single-Center Clinical Study on Unrelated Umbilical Cord Blood Transplantation for the Treatment of Amyotrophic Lateral Sclerosis (ALS)

Study Protocol

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1 Confidentiality Statement

This protocol is provided to investigators as reference material for the clinical trial of unrelated umbilical cord blood transplantation in the treatment of amyotrophic lateral sclerosis (ALS). As it contains confidential information and proprietary technology of the sponsor, it must not be disclosed to any third party other than the investigators and the sponsor without prior authorization. Violators shall be held legally accountable.

2 Research Team

Clinical Team: Zimin Sun, Chief Physician and Ph.D. Supervisor, Regenerative Medicine Diagnosis and Treatment Center. Specializes in the optimization of clinical techniques for umbilical cord blood hematopoietic stem cell transplantation and related basic research. (Team members: Erling Chen, Hong Pan, etc.)

Scientific Research Team: Tao Cheng, Dean, among the first cohort of Tenured Professors and Ph.D. Supervisors at Peking Union Medical College. Engaged long-term in hematology, stem cell biology research, education, and related administrative work. Established the technology system for blood cell reprogramming and gene therapy. (Team members: Sha Hao, Linping Hu, etc.)

Collaborative Team: Yijun Song, Chief Physician and Ph.D. Supervisor, Critical Care Medicine Diagnosis and Treatment Center. Long-term involvement in the diagnosis, treatment, and research of complex neurological diseases such as epilepsy, dementia, and motor neuron diseases. (Team members: Bin Zhang, etc.)

3 Background

3.1 Current Research Status of ALS

ALS, also known as “Lou Gehrig’s Disease”, is a rapidly progressive and fatal neurodegenerative disorder with a global prevalence of approximately 0.73-1.89 per 100,000 individuals [1]. In China, there are an estimated 200,000 ALS patients, with about 25,000 new cases diagnosed annually [1]. The exact etiology of ALS remains

unknown and is currently believed to result from an interplay between genetic and environmental factors. Identified pathogenic genes include *C9orf72*, *SOD1*, *TARDBP*, and *FUS*. The pathogenesis of ALS is complex, with the core feature being the selective loss of motor neurons, involving multiple intertwined pathological processes. Major hypotheses include glutamate excitotoxicity, oxidative stress, protein misfolding and aggregation, and neuroinflammation [2].

Early pathological changes in ALS involve dysfunction of both upper and lower motor neurons and the persistent activation of neuroinflammatory responses. Microglia, the resident immune cells of the central nervous system (CNS), rapidly transition from a resting state to a pro-inflammatory (M1) phenotype. This activation leads to the release of a large number of inflammatory factors (such as TNF-α, IL-1β, IL-6, NO, ROS) and chemokines (such as MCP-1/CCL2), and triggers the NLRP3 inflammasome, directly or indirectly damaging motor neurons. Furthermore, systemic immune dysregulation plays a significant role in ALS pathogenesis. ALS patients exhibit reduced numbers of regulatory T cells (Tregs) in both peripheral blood and the CNS, alterations in the number of activated $CD8^+$ T cell infiltrates, and a shift in the T helper cell (Th1/Th2) balance towards the pro-inflammatory Th1 phenotype, collectively driving the inflammatory microenvironment of the disease [3, 4]. Clinically, elevated inflammatory biomarkers (such as pNfL, sTREM2) are often detected in the cerebrospinal fluid (CSF) of ALS patients, further corroborating the close association between neuroinflammation and ALS disease activity.

Clinically, ALS patients primarily present with progressively worsening muscle weakness, atrophy, fasciculations, and spasticity, ultimately succumbing to dysphagia and respiratory muscle paralysis. Currently, therapeutic options for ALS are extremely limited. In China, only Riluzole and Edaravone are approved for clinical use. Riluzole merely extends patient survival by 2 to 3 months, offering a very modest therapeutic benefit [5]. In recent years, exploratory treatment strategies targeting novel pathways such as neuroinflammation, immune dysregulation, and energy metabolism have

emerged, including the infusion of Tregs, mesenchymal stem cells, and neural stem cells. However, these approaches have still failed to halt disease progression [NCT05695521, NCT03280056, NCT06973629, NCT02290886]. Given the high heterogeneity in ALS pathogenesis and its multisystem involvement, single-target intervention strategies are inadequate to comprehensively correct its widespread functional abnormalities. Therefore, there is an urgent clinical need for novel therapies capable of multi-targeted, systemic intervention.

3.2 Research Progress on Hematopoietic Stem Cell Transplantation (HSCT) for Neuroinflammatory Diseases

Studies have shown that classic chronic neurodegenerative diseases primarily caused by immune dysregulation, such as multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD), all exhibit neuroinflammation. CNS is not an immune-privileged site but rather undergoes immune surveillance under homeostasis. Following injury, various immune cell subsets infiltrate, each exerting distinct and interactive effects on the neural parenchyma [6, 7]. Direct channels exist between the skull bone marrow and the cerebral meninges. After events like stroke or meningitis, these channels can provide a direct pathway, allowing immune cells to be rapidly mobilized from the skull bone marrow into the brain [8]. Professor Qiang Liu's team from the Department of Neurology at Tianjin Medical University General Hospital systematically analyzed the characteristics of bone marrow hematopoiesis in MS patients during the active phase of the disease using techniques such as single-cell sequencing, lineage tracing, and flow cytometry. They discovered that hematopoietic stem cells (HSCs) in MS patients exhibit a myeloid bias. Autoreactive T cells in MS patients home to the bone marrow guided by chemokines like CXCL12. Within the bone marrow, these autoreactive T cells highly express CCL5, leading to the activation and proliferation of HSCs and their downstream myeloid progenitors. This results in abnormal myeloid hyperplasia in the bone marrow, generating a large number of monocytes and neutrophils. These cells can drive T cell-mediated autoimmunity and

migrate to the CNS, causing inflammatory damage [9].

HSCs are among the most widely used and clinically well-established adult stem cell types, serving as the source of all blood cells throughout an organism's lifetime [10]. In recent years, researchers have found that using non-myeloablative conditioning followed by HSCT to treat MS can reboot the immune system and significantly slow the progression of relapsing-remitting MS. Mechanistic studies suggest that HSCT may disrupt the activation cycle between astrocytes and microglia, alleviating the chronic inflammatory state in CNS, while also partially mitigating mitochondrial dysfunction and slowing neurodegeneration [11, 12]. A research team led by Professor Bo Peng from Fudan University and Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine published a breakthrough finding in the journal *Science*. By replacing pathogenic microglia in the CNS through HSCT, they successfully halted disease progression in adult-onset leukoencephalopathy with axonal spheroids and pigmented glia, with patients' motor and cognitive function scores stabilizing or even showing improvement [13]. Given the close association between ALS and neuroinflammation, HSCT holds promise as a crucial avenue for improving the immune environment in ALS and breaking through the current therapeutic bottleneck.

3.3 Advantages of Unrelated Umbilical Cord Blood Transplantation

Unrelated umbilical cord blood transplantation offers advantages such as low HLA matching requirements, a low risk of graft-versus-host disease (GVHD), a potent graft-versus-leukemia (GVL) effect, and immediate availability, leading to its increasingly widespread clinical application [14]. Compared to HSCs from adult donors, cord blood-derived HSCs have lower immunogenicity and allow for HLA mismatches. Typically, transplantation can proceed with an HLA 4/6 match (HLA-A, -B, -DR) between the cord blood unit and the recipient, significantly broadening the donor pool. The immune cells in cord blood are relatively "naïve" and have a weaker ability to recognize and attack recipient tissues, which can significantly reduce the risk of severe GVHD. The

incidence and severity of acute and chronic GVHD after cord blood transplantation are generally lower than those following unrelated matched or mismatched peripheral blood hematopoietic stem cell transplantation. This results in lower transplant-related mortality and allows patients to achieve a better long-term quality of life. Furthermore, unrelated cord blood units are cryopreserved in public or private banks. Once a match is identified, they can be provided promptly, greatly reducing the time needed to find and procure stem cells. This is crucial for ALS patients whose condition is progressing.

To evaluate the safety and efficacy of umbilical cord blood transplantation for ALS patients, investigators plan to conduct an exploratory clinical trial. The preliminary plan is to enroll eight adult subjects. Following successful neutrophil engraftment (defined as an absolute neutrophil count $\geq 0.5\times10^9$/L for three consecutive days) and confirmation of complete donor chimerism, the trial will focus on assessing transplantation-related complications and patient tolerance. The study duration includes subject recruitment, transplantation, and post-transplant follow-up period for comprehensive evaluation. Upon completion of this study, subjects will be invited to continue participating in long-term follow-up (via telephone or hospital visits). This study is a single-arm, single-center, open-label human clinical trial. It will strictly adhere to the Declaration of Helsinki, the International Ethical Guidelines for Biomedical Research Involving Human Subjects by the Council for International Organizations of Medical Sciences, as well as relevant Chinese regulations including the Good Clinical Practice and the Interim Measures for the Administration of Clinical Research on Stem Cells.

To provide clinical investigators with a more comprehensive and in-depth understanding of unrelated umbilical cord blood transplantation for ALS, detailed introductions to the clinical data of the ALS patient cohort, basic experimental results such as drug sensitivity tests, and animal studies are presented herein for clinical trial reference.

4 Clinical Data of the ALS Cohort

4.1 Analysis of Basic Clinical Characteristics Based on Questionnaires from 805 ALS Patients

In the preliminary phase of this study, basic clinical data from 805 ALS patients were collected through an electronic questionnaire system. The data encompassed key indicators such as age, body mass index (BMI), history of smoking and alcohol consumption, past history of chronic diseases, time of ALS onset and diagnosis, type of initial symptoms, and ALSFRS-R scores. Analysis of the results revealed that the patients in this cohort were predominantly middle-aged (concentrated between 45-55 years old). A higher BMI was relatively common, suggesting that overweight or obese body types are somewhat prevalent among ALS patients. The majority of patients (62.33%) presented with limb-onset disease, accompanied by multi-site muscle atrophy. Approximately half (47.83%) of the patients exhibited bulbar symptoms. Some patients had a history of chronic conditions such as hypertension, diabetes, and coronary heart disease. Among these, blood pressure control was generally acceptable overall, while glycemic control levels were generally moderate. A smaller number of patients had a history of rheumatic diseases. Half of the patients experienced sleep disturbances. Almost all patients presented with psychiatric or cognitive symptoms, primarily manifested as low mood and anxiety.

This cohort provides a preliminary sketch of the clinical baseline characteristics of ALS patients in China, offering important epidemiological reference for the subsequent screening of potential patients suitable for HSCT.

4.2 Laboratory Results and In-depth Clinical Assessment of 8 ALS Patients

To further assess the systemic involvement of ALS patients and the feasibility of transplantation, this study adopted a multidisciplinary collaboration model to recruit ALS patients with an ALSFRS-R score $\geq$ 35 points. These patients underwent a systematic, in-depth evaluation primarily encompassing: disease confirmation, immune function testing, bone marrow-related examinations, CSF analysis, and imaging studies.

Specific test items included: complete blood count, liver and kidney function tests, bone marrow morphology, bone marrow flow cytometry, chromosomal analysis, CSF cytokine profiling, electromyography, cardiac ultrasound, pulmonary function tests, magnetic resonance imaging (MRI), and computed tomography (CT). The eight initially enrolled patients were all male, aged between 35 and 48 years, with a time since diagnosis ranging from 5 to 31 months. 75 percent (6 cases) presented with limb-onset symptoms. Two patients had a history of diabetes, and two had a history of hepatitis B. All cases were sporadic. Laboratory tests revealed that the proportion and absolute count of reticulocytes and immature granulocytes in the patients' peripheral blood were elevated, with nearly all patients exceeding the normal reference range (Figure 1). Consistent with this finding, bone marrow flow cytometry results indicated an increased proportion of myeloid lineage cells in 7 patients. Notably, only one patient showed an elevated myeloid cell proportion in the bone marrow smear morphology results. This suggests that flow cytometry may be more sensitive in detecting early or subtle myeloid bias.

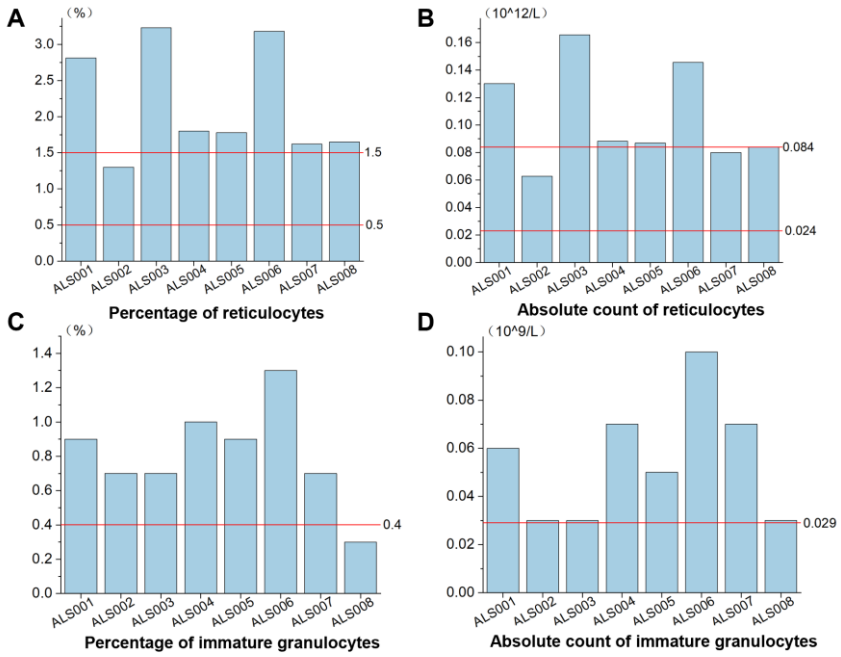
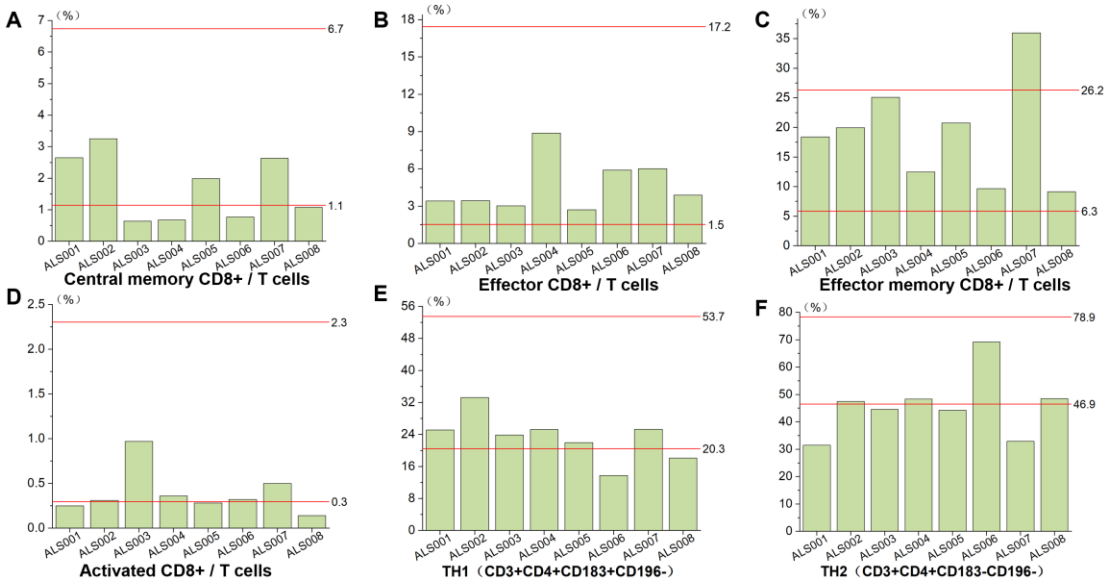


Figure 1. Percentage and absolute of reticulocytes, immature granulocytes in ALS patients

Previous studies have indicated that immune dysregulation plays a significant role in the pathogenesis and progression of ALS. Therefore, we focused on examining the immune function of enrolled ALS patients. Significant abnormalities were observed in the immune cell subsets of all eight patients. Notably, the proportions of naive and effector $CD4^+$ T cells were decreased, while the proportions of activated, effector, and central memory $CD8^+$ T cells were also lower than normal ranges (Figure 2). Furthermore, although peripheral blood levels of inflammatory cytokines showed no significant abnormalities, analysis of CSF cytokine levels in four patients revealed that IL-8 concentrations were more than twice the normal upper limit (Figure 3). This finding indicates the presence of significant neuroinflammation in these ALS patients.



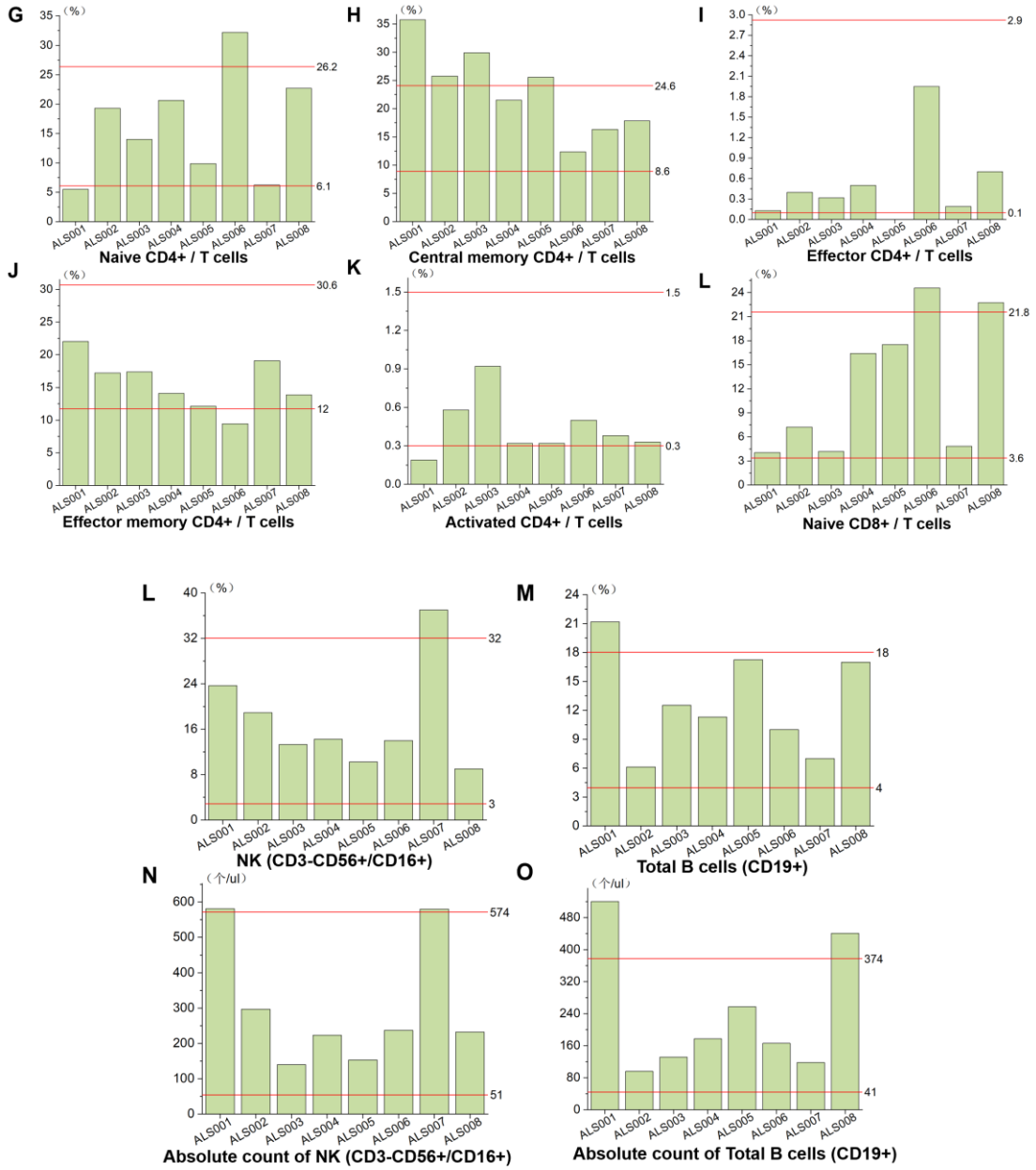


Figure 2. Percentage of immune cells in peripheral blood in ALS patients

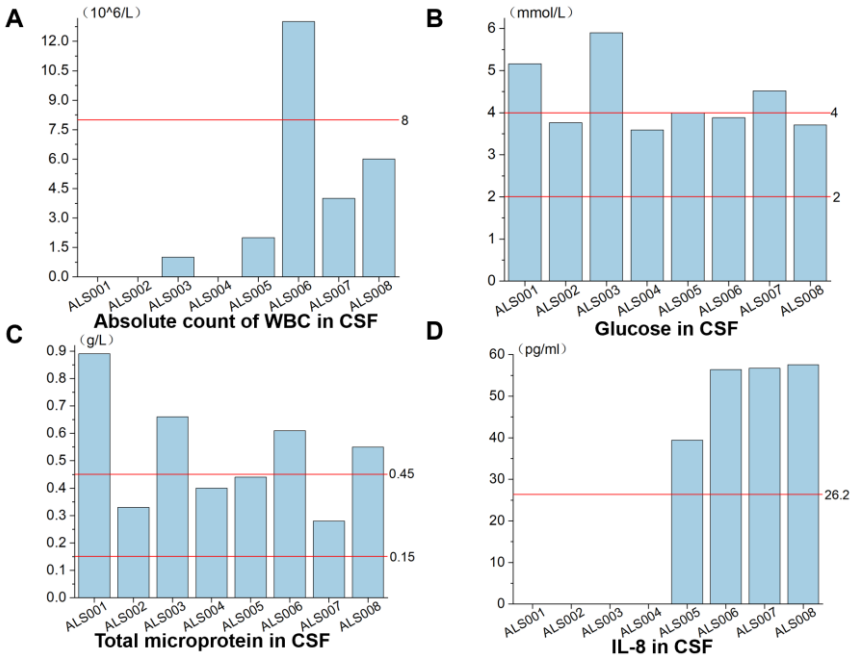


Figure 3. CSF results in ALS patients

In addition, all eight enrolled patients underwent a comprehensive assessment of organ function, including pulmonary function tests, cardiac ultrasound, and vascular ultrasound examinations. Imaging evaluations revealed that all patients exhibited varying degrees of pulmonary imaging abnormalities, suggesting possible interstitial lung involvement. Pulmonary function testing indicated that four patients had moderate restrictive ventilatory dysfunction, with a significant decrease in their measured Forced Vital Capacity (FVC). Four patients were diagnosed with fatty liver via abdominal ultrasound, and three were found to have atherosclerosis with plaque formation based on vascular ultrasound. Cranial magnetic resonance (MR) imaging showed that four patients had intracranial demyelinating lesions. Furthermore, one patient was diagnosed with deep vein thrombosis in the lower limbs and subsequently initiated on anticoagulant therapy with Rivaroxaban. These collective findings reflect the frequent multisystem involvement and comorbidities in ALS patients, providing crucial clinical evidence for screening patients suitable for HSCT in this study. Considering the specific requirements of HSCT for overall organ reserve capacity, particularly pulmonary function, this series of assessments preliminarily identified a patient subgroup capable

of tolerating transplantation-related stress. For individuals whose key indicators, such as pulmonary function, do not meet transplantation criteria, further comprehensive evaluation by a multidisciplinary team is required to assess the associated treatment risks and benefits.

5 Experimental Results

5.1 Single-Cell Sequencing

Compared to healthy donors, the bone marrow of ALS patients contained a lower proportion of hematopoietic stem and progenitor cells (HSPCs), particularly HSCs and multipotent progenitors (MPPs). Furthermore, the overall HSC/MPP population in ALS patients exhibited characteristics closer to long-term HSCs (LT-HSCs), with significantly weaker lineage commitment, particularly toward myeloid and lymphoid lineages. An increasing trend was observed in the proportion of B-cell precursors within the bone marrow HSPCs of ALS patients. Additionally, analysis of mature cell populations revealed divergent trends in myeloid and lymphoid subsets between peripheral blood and bone marrow in ALS patients. In bone marrow, the proportion of monocytes was relatively low, while that of $CD4^+$ T cells was relatively high. This suggests a potential inflammatory state within the bone marrow, possibly promoting the homing of peripheral $CD4^+$ T cells to this site. Conversely, in peripheral blood, the proportions of monocytes and $CD8^+$ T cells were elevated (Figure 4).

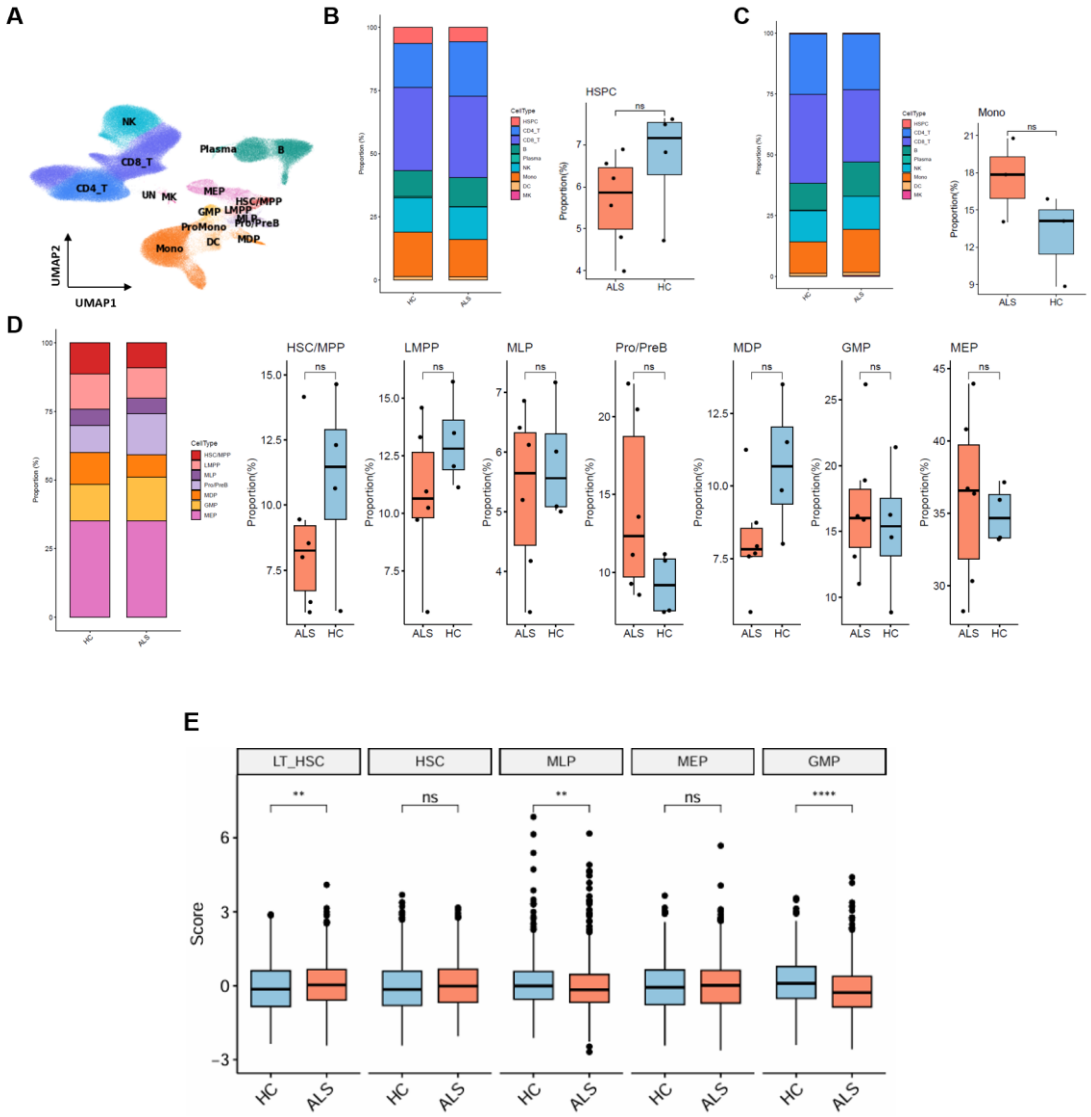


Figure 4. Comparison of cell subtypes between ALS and healthy control based on RNA-seq

5.2 Flow Cytometry Results

In peripheral blood, ALS patients exhibit a decreased proportion of T cells and an increased proportion of myeloid cells in peripheral blood based on flow cytometry analysis, although these differences did not reach statistical significance. In contrast, within bone marrow, ALS patients showed a significantly higher proportion of T cells and a significantly lower proportion of myeloid cells compared to healthy controls (Figure 5). The underlying mechanisms driving these distinct cellular compositional differences between bone marrow and peripheral blood in ALS patients require further

investigation.

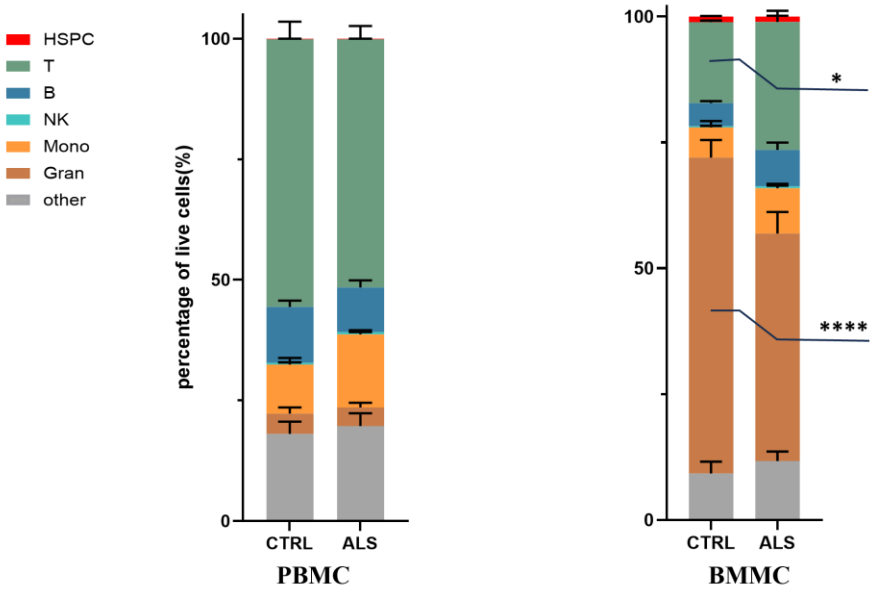


Figure 5. Comparison of cell subtypes between ALS and healthy control based on flow cytometry

5.3 Colony-Forming Cell (CFC) Assay

In CFC assay, investigators compared the ability of $CD34^+$ cells from ALS and control groups to form various clonal colonies in semi-solid culture medium. Primary CFC assay showed a declining trend in the number of CFU-GM colonies formed by the same quantity of cells from the experimental group. However, the secondary CFC assay indicated an increase in myeloid lineage colonies in the patients. These results suggest that HSPCs from ALS patients may experience a differentiation block (Figure 6). Furthermore, investigators observed that within the ALS experimental group, as the patients' condition worsened, the colony-forming capacity of their HSPCs progressively declined. The number of clonal colonies was significantly lower in ALS patients who had already developed paralysis.

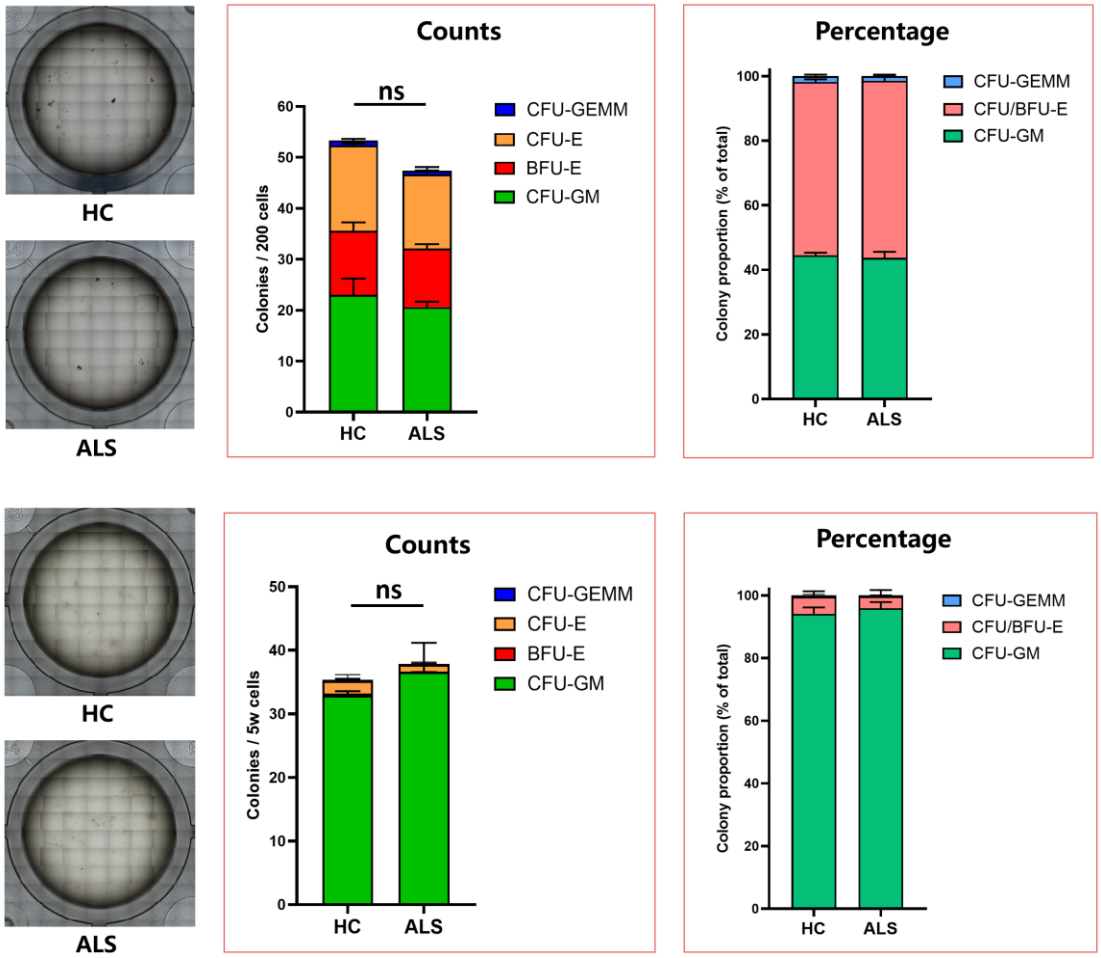


Figure 6. CFC assay (primary, second)

5.4 Drug Sensitivity Testing

Prior to undergoing HSCT, ALS patients require pharmacological or radiotherapeutic intervention to eliminate aberrant microglia and other cells. Based on findings from previous research, investigators designed drug sensitivity assays to evaluate the efficacy of different agents in killing microglial cells. The results demonstrated that the combination of Melphalan and Fludarabine exhibited superior efficacy in eliminating microglial cells, followed by Thiotepa (Figure 7). This provides a theoretical basis for the myeloablative conditioning regimen prior to transplantation.

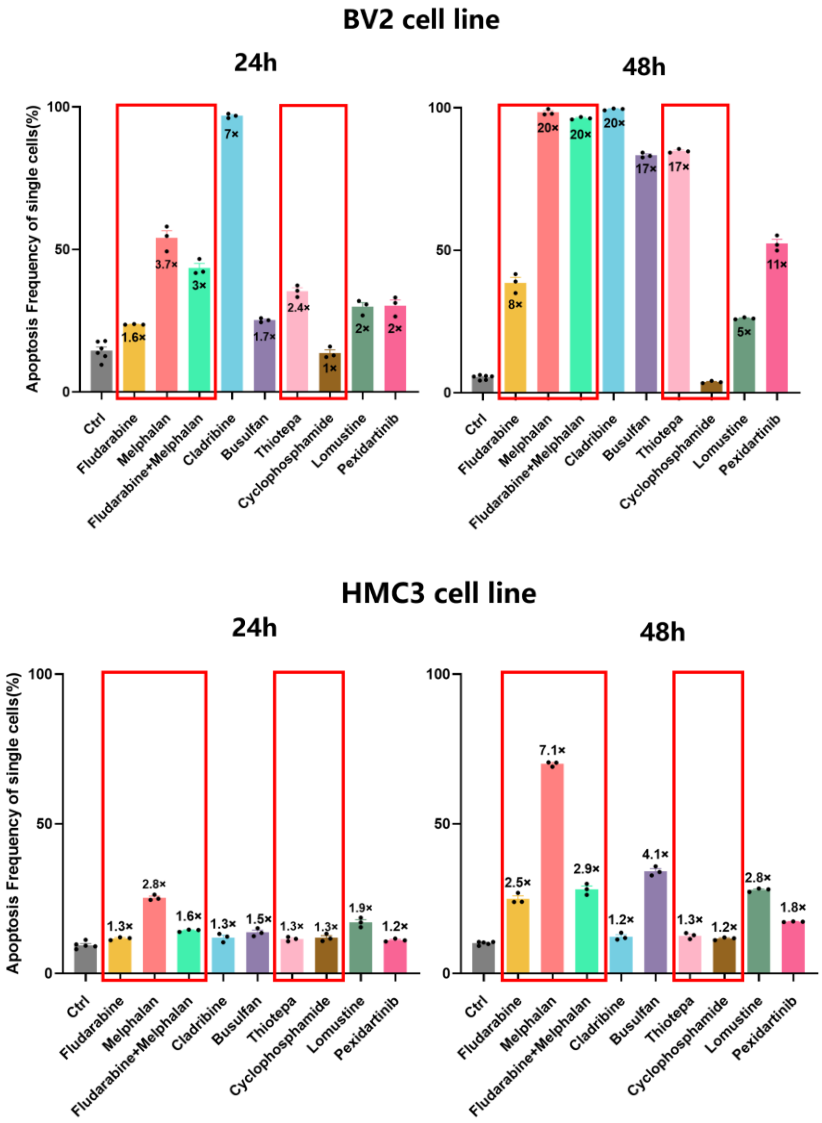


Figure 7. Drug sensitivity test

6 Animal Experimental Data

Research conducted by the team at the University of Pittsburgh, Epperly et al. (2019), demonstrated that HSCT combined with myeloablative conditioning significantly extended paralysis-free survival in the SOD1G93A mouse model, a widely recognized model of ALS. The research team used SOD1G93A mice that had developed grade I paralysis at 90 days of age. The mice received a total body irradiation (TBI) dose of 9 Gy (Cesium source, 340 cGy/min), followed by intravenous injection of 1×10^6 C57BL/6 GFP^+ donor bone marrow cells. The results showed that the mice's

survival was extended from 129 days to 233 days (p=0.0045), and the paralysis-free period was prolonged to over 250 days. The donor-derived bone marrow cells differentiated into M2 microglia, exerting a neuroprotective effect by localizing to the degenerating motor neuron regions in the spinal cord anterior horn. These M2 microglia likely inhibited neuroinflammation through the secretion of neurotrophic factors or anti-inflammatory cytokines [15]. Traditional non-gene therapies (such as antioxidants, anti-inflammatory drugs, and neurotrophic factors) only extended the survival of SOD1 mice by 6-10 days, showing a minimal effect. The transplantation strategy employed by Epperly et al. extended the survival of ALS mice by over 100 days, demonstrating significant therapeutic potential.

7 HSCT Protocol and Associated Risks

7.1 Inclusion Criteria

(1) Patients with a confirmed diagnosis of ALS, with an ALSFRS-R score ≥ 35 and a maximum vital capacity ≥ 65%

(2) Aged > 35 years and < 50 years, with no gender restrictions

(3) Karnofsky Performance Status (KPS) score ≥ 70, and Eastern Cooperative Oncology Group (ECOG) Performance Status ≤ 2

(4) Unrelated umbilical cord blood and the recipient demonstrate high-resolution HLA (-A, -B, Cw, DR, DQ) matching at ≥ 4/6, 5/8, or 6/10 loci, and the post-thaw $CD34^+$ cell count in the cord blood unit is ≥ 1.2×10^5/kg (recipient body weight)

(5) Willing and able to comply with the study procedures and conditions, demonstrating good compliance

(6) Willing to receive at least two years of treatment and follow-up, with detailed medical records maintained

(7) Patients and/or their legal guardian voluntarily participate in this clinical trial,

sign the informed consent form, and are capable of completing all follow-up assessments as required by the protocol

7.2 Exclusion Criteria

(1) Patients with positive results in the following etiological tests: Human Immunodeficiency Virus (HIV-1/2), Human Cytomegalovirus DNA (HCMV-DNA), Epstein-Barr Virus DNA (EBV-DNA), Hepatitis B (positive for Hepatitis B surface antigen (HBsAg) or Hepatitis B virus DNA (HBV-DNA)), Hepatitis C antibody (HCV-Ab), or Treponema pallidum antibody (TP-Ab)

(2) Clinically significant active bacterial, viral, fungal, or parasitic infections as judged by the investigator during screening

(3) Patients who have previously received gene therapy or allogeneic hematopoietic stem cell transplantation

(4) First-degree relatives with known or suspected familial cancer syndromes (including but not limited to hereditary breast and ovarian cancer syndrome, hereditary nonpolyposis colorectal cancer syndrome, familial adenomatous polyposis, etc.)

(5) Diagnosis of major psychiatric disorders or predisposition to such conditions that would significantly impair the ability to participate in the clinical study

(6) History of major organ impairment, including: Liver disorders: Liver function tests showing AST or ALT > 3 × ULN; total serum bilirubin > 2.5 × ULN; for cases consistent with Gilbert syndrome, total bilirubin > 3 × ULN and direct bilirubin > 2.5 × ULN; history of hepatic bridging fibrosis, cirrhosis, or active hepatitis; Cardiac disorders: Left ventricular ejection fraction (LVEF) <45% at screening; New York Heart Association (NYHA) Class III or IV congestive heart failure; severe arrhythmias requiring treatment; poorly controlled hypertension (systolic blood pressure > 160 mmHg and/or diastolic blood pressure > 100 mmHg despite antihypertensive therapy), or prior history of hypertensive emergencies, hypertensive encephalopathy, or unstable angina; history of myocardial infarction, coronary artery bypass graft surgery,

peripheral arterial bypass graft implantation, or stent placement within 12 months prior to enrollment; clinically significant valvular disease; calculated eGFR < 60 mL/min/1.73m²; Pulmonary function: FEV1/FVC < 60% and/or diffusing capacity less than 60% of predicted; clinically significant evidence of pulmonary hypertension requiring medical intervention

(7) Uncorrectable coagulation dysfunction or history of severe bleeding disorders

(8) Any other condition deemed by the physician to render the subject unsuitable for hematopoietic stem cell transplantation

(9) Known hypersensitivity to the investigational drug or its components

(10) Participation in or ongoing participation in other interventional clinical studies within 3 months prior to screening

(11) Vaccination with live vaccines within 6 weeks prior to screening

(12) Pregnant or breastfeeding women

(13) History of solid organ transplantation

(14) Poor compliance of the subject with the study protocol

(15) Any other condition considered by the investigator as unsuitable for participation in this clinical trial

(16) Unwillingness of the subject to provide pre-existing valid diagnostic evidence before treatment or to undergo bone marrow, lumbar puncture, blood tests, and other examinations after treatment

7.3 Myeloablative Conditioning Regimen and GVHD Prophylaxis Protocol

(I) Reduced-Intensity Myeloablative Conditioning Regimen

Based on preliminary research and the results of drug sensitivity assay, this study employs a reduced-intensity myeloablative conditioning regimen (conditioning intensity score TCI = 3.5) consisting of Fludarabine (Flu), Melphalan (Mel), Thiotepa

(TT), and Total Marrow Irradiation (TMI). The goal is to maximally eliminate abnormally activated immune cells and potentially pathogenic microglial cells, thereby creating the necessary immune and bone marrow niche for umbilical cord blood stem cell engraftment.

The specific drug administration schedule is as follows:

TT: Total dose: 10 mg/kg. Administered at 5 mg/kg/day, qd via intravenous infusion (iv) on Days -6 and -5.

Flu: Total dose: 150 mg/m². Administered at 30 mg/m²/day, qd, iv from Day -6 to Day -2.

TMI: Total dose: 400 cGy. Administered as a single dose of 400 cGy on Day -2.

Mel: Total dose: 100 mg/m². Administered qd, iv on Day -1.

(Note: Doses will be adjusted based on the patient's renal and hepatic function during implementation)

Considering that the conditioning process may induce significant oxidative stress, the antioxidant food supplement sulforaphane will be co-administered prophylactically to mitigate potential oxidative damage. Preliminary clinical studies have indicated that sulforaphane not only possesses antioxidant properties but may also promote umbilical cord blood stem cell engraftment. Sulforaphane will be administered orally at a dose of 1 tablet, three times daily, starting from the beginning of the conditioning regimen (Day -6) until Day +7 post-transplantation. If the umbilical cord blood unit used has been cryopreserved for > 10 years, the sulforaphane dose will be increased to 2 tablets, three times daily, from Day -1 to Day +7.

(II) GVHD Prophylaxis Protocol

GVHD prophylaxis will be implemented using a combination of Cyclosporine A (CsA) and short-course Mycophenolate Mofetil (MMF). CsA: Initial dose of 2.5 mg/kg/day. Administration will begin on Day -1 (one day before transplantation) as a

continuous 24-hour intravenous infusion. The target therapeutic blood concentration will be maintained between 200-300 ng/mL. After neutrophil engraftment and recovery of gastrointestinal function, CsA will be switched to oral administration at twice the intravenous dose. The target trough concentration will be maintained at 150-200 ng/mL until 2 months post-transplantation. The dose will be adjusted based on the presence of GVHD, infection status, and patient tolerance. CsA will be gradually tapered starting at 2 months post-transplantation and completely discontinued by 5-6 months. MMF: Dose of 25-30 mg/kg/day. Administration will commence on Day +1. The dose will be gradually reduced following neutrophil engraftment and completely discontinued by 3 months post-transplantation.

7.4 Unrelated Umbilical Cord Blood Transplantation Protocol

(I) Criteria for Determining a Single Umbilical Cord Blood Unit

(1) HLA matching between donor and recipient should ideally be ≥ 4/6, 5/8, or 7/10 (high-resolution genotyping).

(2) Post-thawing (from a small segment), the cord blood unit must have a total nucleated cell (TNC) count ≥ 1.5×10^7/kg and a $CD34^+$ cell count ≥ 1.2×10^5/kg. The CFU-GM count should correlate with the $CD34^+$ cell count or demonstrate good colony formation. For patients in urgent need of transplantation, cord blood units with a $CD34^+$ cell count ≥ 0.83×10^5/kg may be considered.

(3) Donor-specific antibodies (DSA) must be negative (against 12 HLA loci).

(4) Donor-recipient gender: No specific requirement for single-unit umbilical cord blood transplantation. Donor-recipient ABO blood type: No specific requirement for single-unit umbilical cord blood transplantation.

(II) Mobilization and Collection of Recipient's Autologous Peripheral Blood Hematopoietic Stem Cells during the Screening Phase for Cryopreservation and Future Use

(1) Use a Granulocyte Colony-Stimulating Factor (G-CSF) mobilization protocol to stimulate the release of hematopoietic stem cells from the bone marrow into the peripheral blood: G-CSF 5 μg/kg, administered subcutaneously every 12 hours (q12h).

(2) Timing of collection: Monitor complete blood count daily after mobilization. The $CD34^+$ cell peak typically occurs 4-6 days after G-CSF mobilization. Collection begins when the white blood cell count exceeds 5×10^9/L and the platelet count exceeds 50×10^9/L.

(3) Stem cell collection: Use a cell separator to collect peripheral blood stem cells. The minimum target $CD34^+$ cell dose is $\geq 2 \times 10^6$/kg (recipient body weight), with an ideal target of $\geq 5 \times 10^6$/kg. Strictly monitor electrolytes during the collection process.

(4) Stem cell cryopreservation: Use a cell freezing solution containing the cryoprotectant dimethyl sulfoxide (DMSO) (maximum final DMSO concentration should not exceed 10%). After controlled-rate freezing, the autologous hematopoietic stem cells are cryopreserved in liquid nitrogen at -196°C.

(III) Umbilical Cord Blood Infusion

On transplant day 0, the thawed umbilical cord blood unit is infused. Dexamethasone 5 mg is administered intravenously 30 minutes prior to infusion to prevent infusion-related reactions. Under continuous and close monitoring of vital signs, the thawed cord blood hematopoietic stem cell suspension is infused at a constant rate via a central venous catheter. Following the infusion, approximately 100 mL of normal saline is slowly dripped to flush the infusion line, ensuring all cells are delivered into the patient's circulation.

7.5 Post-Transplant Monitoring and Management of Potential Complications

Post-transplantation management, as the core safety component of this study's implementation, focuses on risks arising from conditioning toxicity, HSCT-related complications, and the progression risk of the underlying ALS disease. Specific aspects include:

(1) Conditioning-Related Toxicities: Primarily manifest as pancytopenia due to myelosuppression, leading to risks of secondary infections and bleeding. Other potential toxicities include mucositis, organ function impairment, and electrolyte imbalances. Active supportive care is essential, encompassing blood product transfusions, anti-infective therapy, nutritional support, and organ function support.

(2) Infusion-Related Adverse Reactions: The cryoprotectant DMSO contained in the cord blood product may cause a range of reactions. Common symptoms include nausea, vomiting, abdominal pain, bradycardia, blood pressure fluctuations, urticaria, and other allergic manifestations. Severe cases may present with dyspnea or hemoglobinuria. Management principles involve timely symptomatic intervention, such as enhanced hydration and diuresis, antihistamines for pruritus, and antiemetics/sedatives. Infusion should be paused and emergency medical support initiated for severe reactions.

(3) GVHD: Although the incidence of acute and chronic GVHD is relatively lower after cord blood transplantation, risk remains. Typical acute GVHD may present with fever, skin rash, jaundice, and diarrhea. A calcineurin inhibitor-based immunosuppressive regimen (e.g., tacrolimus) will be employed for prophylaxis, with close monitoring for relevant clinical signs and laboratory indicators.

(4) Rescue Protocol for Graft Failure: Primary graft failure is defined as the failure to achieve neutrophil engraftment by day 28 post-transplantation. Complete blood counts will be monitored daily post-transplant. Donor-recipient chimerism status will be dynamically assessed using STR-PCR (peripheral blood samples on days 7, 14, 21; bone marrow sample on day 28). A donor chimerism rate < 50% on day 21 suggests a risk of delayed engraftment. A diagnosis of primary graft failure is made if neutrophils are not engrafted by day 28 and the bone marrow donor chimerism rate is < 50%. Autologous peripheral blood HSCs mobilized and collected with G-CSF during the screening phase and cryopreserved for backup will be reinfused to restore hematopoietic function upon confirmation of primary graft failure.

(5) Delayed Immune Reconstitution: T-cell recovery: Reconstitution of cord blood-derived naïve T cells is slow. Close monitoring for CMV/EBV reactivation is required. Letermovir will be routinely administered from day +7 for CMV prophylaxis. B-cell function: Hypogammaglobulinemia will be managed with intravenous immunoglobulin replacement therapy.

(6) Risk of Infection Transmission: Although all cord blood units undergo rigorous screening per national standards for pathogens including HIV, HBV, HCV, and syphilis, and are assessed for bacterial contamination and xenotransplantation-related infection risks, a minimal possibility of transmission of unknown or latent pathogens cannot be entirely excluded. Systemic infection monitoring and prophylaxis will be implemented post-transplantation.

(7) Progression of Underlying Disease During Transplantation and Countermeasures: Despite the strict inclusion criterion of FVC ≥ 65% predicted in this study, given that ALS is a progressively deteriorating disease, there remains a risk of significant pulmonary function decline during the transplantation process. Prior to transplantation, patients will undergo non-invasive ventilation (NIV) tolerance training, and individualized ventilation plans will be established. Continuous monitoring of ECG, respiration, and blood pressure will be maintained throughout the process, with arterial blood gas analysis performed as needed. Should acute or subacute respiratory deterioration occur, a pre-defined respiratory support protocol will be immediately initiated, escalating from supplemental oxygen and NIV to, if necessary, invasive mechanical ventilation, to ensure patient safety. This entire management process will be jointly executed by a multidisciplinary team comprising Neurology, Critical Care Medicine, and Anesthesiology departments to minimize risks posed by ALS progression during transplantation.

8 Evaluation

To comprehensively assess the safety of unrelated umbilical cord blood

transplantation for the treatment of ALS, this study will employ multidimensional assessment tools to evaluate participants. Safety assessments will be conducted throughout the entire study period, involving continuous monitoring and documentation of all adverse events and serious adverse events. Particular attention will be paid to the progression and evaluation of ALS, as well as transplantation-related complications such as GVHD, infections, graft failure, and organ toxicities associated with the conditioning regimen.

8.1 Safety Assessment System

(1) LVGI Safety Score

Considering the limited assessment conditions within the transplant isolation unit, this study has modified and simplified the ALSFRS-R scale and incorporated the Inbody score, which provides a comprehensive assessment of muscle mass and body fat mass, to form the "LVGI" safety score (details in the table below). Among the components, VCmax refers to maximum lung capacity; Grip quantifies upper limb strength using a dynamometer, with the Grip Strength-to-Body Weight Index calculated as Grip Strength (kg) / Body Weight (kg) × 100. The LSGI assessment is performed weekly before transplantation and during the transplantation process for the patient. The results of each assessment are compared. A change within ± 20% in the LSGI score within the first month post-transplantation compared to the pre-transplantation score is considered safe.

Part	**Item**	**Score**
Medulla Function	Language	4 Normal 3 Detectable speech impairment 2 Comprehensible repetitive speech 1 Speech is non-communicative 0 Absence of effective speech
Respiratory	VCmax	4 VCmax > 80%

Function		3 70% < VCmax ≤ 80% 2 60% < VCmax ≤ 70% 1 50% < VCmax ≤ 60% 0 VCmax ≤ 50%
Upper Limb Function	Grip Strength-to-Body Weight Index	4 Grip > 40 3 30 < Grip ≤ 40 2 20 < Grip ≤ 30 1 10 < Grip ≤ 20 0 Grip ≤ 10
Overall Muscle Status	Inbody Score	4 Inbody > 80 points 3 70 points < Inbody ≤ 80 points 2 60 points < Inbody ≤ 70 points 1 50 points < Inbody ≤ 60 points 0 Inbody ≤ 50 points

(2) Assessment of Drug-Induced Liver and Kidney Toxicity

The Roussel Uclaf Causality Assessment Method (RUCAM) will be used to assess the occurrence of drug-induced liver injury; Kidney Disease Improving Global Outcomes criteria for Acute Kidney Injury will be used to assess the occurrence of drug-induced kidney injury.

(3) Assessment of acute GVHD

The grading criteria for the severity of acute GVHD are established based on its impact on non-relapse mortality post-transplantation. This involves separate scoring for acute GVHD affecting the skin, gastrointestinal tract, and liver, which are then combined to determine an overall severity grade. The assessment primarily follows the modified Glucksberg criteria and the grading system established by the Mount Sinai Acute GVHD International Consortium.

8.2 Multidimensional Efficacy Assessment

Overall Survival (OS): Defined as the time from umbilical cord blood infusion to death due to any disease-related cause. This endpoint is used to assess the fundamental impact of umbilical cord blood transplantation on patient survival.

Assessment of Disease Functional Progression: Standardized evaluation using the ALSFRS-R scale will be conducted at baseline, as well as at 1, 2, 3, 6, 12 and 24 months post-transplantation. Changes in scores over time will be analyzed.

Biomarker of Neuroaxonal Injury: Peripheral blood and CSF samples will be collected to assess levels of NFL. The dynamic changes in neuroaxonal injury following umbilical cord blood transplantation intervention will be objectively evaluated by analyzing changes in NFL relative to baseline.

Imaging Evaluation: A combined approach using MRI and PET-CT will be employed to quantify the degree and rate of upper motor neuron degeneration. Specific tracers targeting activated microglia will be used to quantitatively analyze changes in neuroinflammatory levels in key areas such as the motor cortex and brainstem.

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