Clinical Research Protocol

Protocol Title:	Efficacy and Safety Study of Umbilical Cord Blood Transplantation (UCBT) with Total Marrow Irradiation (TMI)-Based Conditioning Regimen for Adults with Refractory/Relapsed Aplastic Anemia (AA)
Protocol Number:	BDHAP-TMIUCBT-20240228
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Confidentiality Statement

Investigator's Statement:

I am familiar with this research protocol and confirm that the protocol contains the necessary content for the implementation of the research and the relevant responsibilities associated with this research protocol. I agree to comply with the relevant responsibilities in accordance with Chinese laws and regulations, the Declaration of Helsinki, Good Clinical Practice for Drug Clinical Trials, and this research protocol. The research procedures specified in this protocol may only be carried out after the ethics committee has approved the protocol and the subjects have provided informed consent. Any modifications to the protocol must be approved by the ethics committee before implementation, unless measures must be taken to protect the safety, rights, and interests of the subjects. I understand and comply with the requirements for the maintenance of original data.

Principal Investigator's Name: Sun zimin

Date: May 28, 2025

Clinical Research Institution:nstitute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College

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Abbreviations

Abbreviation	English Term
CRF	Case Report Form
V/SAA	Very/Severe Aplastic Anemia
aGVHD	Acute Graft-versus-Host Disease
Allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplantation
ANC	Absolute Neutrophil Count
ATG	Antithymocyte Globulin
Bu	Busulfan
CB	Cord Blood
CBT	Cord Blood Transplantation
cGVHD	Chronic Graft-versus-Host Disease
CSA	Cyclosporine
CY	Cyclophosphamide
DFS	Disease-Free Survival
DSA	Donor Specific Antibody
EFS	Event-Free Survival | Event-Free Survival
ES	Engraftment Syndrome
Flu	Fludarabine
FK506	Tacrolimus
GF	Graft Failure
GVHD	Graft-versus-Host Disease
Haplo-HSCT	Haploidentical Hematopoietic Stem Cell Transplantation
HLA	Human Leukocyte Antigen
HGB	Hemoglobin | Hemoglobin
HRQOL	Health-Related Quality of Life
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
HVOD	Hepatic Veno-Occlusive Disease
IA	Invasive Aspergillosis
IFD	Invasive Fungal Disease
IFN	Interferon | Interferon
IRM	Infection-Related Mortality
IST	Immunosuppressive Therapy
KIR	Killer-Cell Immunoglobulin-Like Receptor
MAC	Myeloblative Regimen
Mel	Melphalan
MFD	Matched Family Donor
MHC	Major Histocompatibility Complex
MMF	Mycophenolate Mofetil
MSD	Matched Sibling Donor

MTX	Methotrexate
MUD	Matched Unrelated Donor
NGS	Next-Generation Sequencing
NMC	Nonmyeloablative Regimen
OM	Oral Mucositis
OS	Overall Survival
PRA	Population Reactive Antibody
PES	Pre-Engraftment Syndrome
PLT	Platelet
PNH	Paroxysmal Nocturnal Hemoglobinuria
R-V/SAA	Refractory Very/Severe Aplastic Anemia	R
SAA	Severe Aplastic Anemia
SCT	Stem Cell Transplantation
STR	Short Tandem Repeat
TA-TMA	Transplant-Associated Thrombotic Microangiopathy
TMA	Thrombotic Microangiopathy
TMI	Total Marrow Irradiation
TNC	Total Nucleated Cell
TNF	Tumor Necrosis Factor
UCB	Umbilical Cord Blood
UCBT	Umbilical Cord Blood Transplantation
UPBSCT	Unrelated Peripheral Blood Stem Cell Transplantation
WBC	White Blood Cell
CR	Complete Response
PR	Partial Response
NR	No Response
RFS	Relapse-Free Survival
SAE	Serious Adverse Event

1.Overview

Protocol Overview Date: May 28, 2025
Research Drug Name: Total Marrow Irradiation (TMI)-Based Conditioning Regimen
Clinical Trial Phase:Phase II Clinical Study
Title: Efficacy and Safety Study of Umbilical Cord Blood Transplantation (UCBT) with Total Marrow Irradiation (TMI)-Based Conditioning Regimen for Adults with Refractory/Relapsed Aplastic Anemia (AA)
Planned Study Period: June 2025 to June 2028
Study Objectives： （1）Primary Objective: To evaluate the efficacy of UCBT with TMI-based conditioning regimen for adults with refractory/relapsed AA. （2）Secondary Objective: To assess the safety of the regimen.
Study Population: Adults with refractory/relapsed AA
Planned Number of Subjects: 11 subjects
Study Design Overview: This is a Phase II clinical study. A total of 11 adult patients with refractory/relapsed AA will be treated with a UCBT regimen based on a TMI-based conditioning regimen. Patients who meet the inclusion/exclusion criteria will sign an informed consent form before undergoing cord blood transplantation. The efficacy (12-month EFS) and safety of the regimen will be assessed 12 months after transplantation.
Inclusion Criteria: Subjects suitable for enrollment in this study must meet all of the following criteria: 1. Age ≥18 years and <50 years, regardless of gender. 2. Diagnosis of AA according to the guidelines of the British Society for Haematology. The bone marrow shows hypocellularity, without infiltration of abnormal cells or marrow fibrosis. The peripheral blood must meet at least two of the following three criteria: absolute neutrophil count (ANC) <1.5×10^9 /L; platelet count <50×10^9 /L; hemoglobin <100 g/L. Based on the severity of the disease, AA is classified into severe aplastic anemia (SAA) and non-severe aplastic anemia (NSAA). The diagnostic criteria for severe aplastic anemia (SAA) are as follows: Peripheral blood criteria At least two of the following three criteria must be met: Absolute neutrophil count (ANC) <0.5×10^9 /L; Reticulocyte count <20×10^9 /L; Platelet count <20×10^9 /L; Bone marrow criteria The degree of bone marrow cellularity is <25% of normal; if ≥25% but <50% of normal, then the proportion of residual hematopoietic cells should be <30%. Bone marrow aspiration results show hypocellular or severely hypocellular marrow, with a marked decrease in hematopoietic cells and an increase in non-hematopoietic cells such as lymphocytes and reticular cells. Other： Other diseases causing pancytopenia, such as myelodysplastic syndrome (MDS),

paroxysmal nocturnal hemoglobinuria (PNH), and congenital marrow failure syndromes, should be excluded.

If ANC <0.2×10^9 /L, the diagnosis is very severe aplastic anemia (VSAA);

Those who do not meet the criteria for SAA are classified as non-severe aplastic anemia (NSAA);

3. Meet the criteria for refractory AA: ①SAA: no response to first-line ATG + CSA treatment for more than 6 months; ②NSAA: ①persistent transfusion dependence of two or more blood cell lines, ②meet any of the following criteria: no response to immunosuppressive therapy with cyclosporine for more than 12 months, or no response to androgen and/or TPO receptor agonist therapy for more than 6 months, ④or progression to SAA;

Meet the criteria for relapsed AA: initially effective to first-line immunosuppressive therapy, but pancytopenia recurs after reduction or discontinuation of immunosuppressive therapy, meeting the criteria for SAA.

4. Karnofsky score ≥60, ECOG score ≤2, HCT-CI index ≤2.

5. No HLA-identical sibling donor or no HLA-identical unrelated donor available.

6. Understanding of the study procedures and voluntary written informed consent.

Exclusion Criteria:

Subjects with any of the following conditions are not eligible for enrollment in this study:

1. Pancytopenia and hypoplastic marrow diseases caused by other reasons (including PNH, etc.).

2. Use of intermediate or high doses of cyclophosphamide (≥ 20mg/kg/d) for immunosuppressive therapy within 3 months before enrollment.

3. History of hematopoietic stem cell transplantation.

4. Known or suspected contraindications or allergies to fludarabine, melphalan, or other drugs.

5. Patients with uncontrolled bleeding and/or infection after standard treatment before screening.

6. Active viral hepatitis (hepatitis B, hepatitis C, etc.), HIV infection, or syphilis at baseline or screening.

7. Creatinine clearance rate <60 ml/min before treatment, or serum creatinine >140 μmol/L.

8. Pregnant or breastfeeding women.

9. Participation in another clinical trial within 3 months before enrollment.

10. Any other condition that the investigator deems may prevent the subject from completing the study or pose significant risks to the subject.

Study Protocol: TMI-Based Conditioning Regimen

Medication Regimen:

1. Conditioning Regimen:

- Flu 30 mg/m^2 × 5 days on Days -6, -5, -4, -3, -2;
- TMI 4 Gy qd on Day -2;
- Mel 100 mg/m^2 qd on Day -1.

2. Single Cord Blood Unit:

- HLA Matching: The cord blood unit should have an HLA match of ≥4/6, 5/8, 6/10 with the patient.
- DSA: Within 2 months before transplantation, patients will undergo anti-HLA antibody testing to select a DSA-negative cord blood unit.

- Target Cord Blood Quality: The total nucleated cell (TNC) count should be $\geqslant 2\times10^{7}$ /kg (patient weight) before freezing, CD34+ cells should be $\geqslant 1.0\times10^{5}$ /kg (patient weight), and the CD34+ cell count should be $\geqslant 0.83\times10^{5}$ /kg (patient weight) after thawing.

3. GVHD Prophylaxis:

- Cyclosporine 2.5 mg/kg/d continuous intravenous infusion starting on Day -1, which can be switched to oral administration after hematopoietic reconstitution or recovery of gastrointestinal function, with a dose twice that of the intravenous infusion, maintaining cyclosporine blood concentration between 200-300 ng/ml;

(Alternative FK506 0.03 mg/kg continuous 24-hour intravenous infusion or oral administration starting on Day -1, with oral dosage being twice that of the intravenous infusion, divided into two doses).

- Mycophenolate mofetil 30 mg/kg/d, intravenous infusion (or oral), divided into two doses, starting on Day +1, gradually tapered with myeloid reconstitution, and discontinued around Day +60.

Concomitant Therapies

1. Complications Prevention

- VOD Prevention: Ursodeoxycholic acid 250 mg tid orally, starting with conditioning regimen;

Prostaglandin E1 10 μg qd intravenous injection, starting with conditioning regimen (discontinued on Day +30);

Heparin 100 IU/kg/d intravenous infusion, starting with conditioning regimen (discontinued when platelet count is below 20×10^{9}/L).

- CMV Prevention: In accordance with relevant guidelines, patients positive for CMV-IgG will receive letermovir starting on Day +7, discontinued after 3 months.

- Herpes Virus Prevention: Acyclovir 10 mg/kg/d divided into two doses, starting on Day +1, discontinued after 1 year.

- Fungal Prevention: In accordance with relevant guidelines, primary prophylaxis for IFD will be initiated concurrently with the pre-transplant conditioning regimen for patients undergoing UCBT, lasting at least until 3 months post-transplant. If acute or chronic GVHD or immunosuppressive therapy is present, the duration will be extended until clinical control of GVHD and discontinuation of immunosuppressive agents. Triazole or echinocandin antifungal agents will be used for primary prophylaxis. For patients with a history of confirmed or clinically diagnosed IFD, antifungal agents will be used for secondary prophylaxis after IFD has achieved complete or partial remission, lasting at least until 3 months post-transplant. If acute or chronic GVHD or immunosuppressive therapy is present, the duration will be extended until clinical control of GVHD and discontinuation of immunosuppressive agents.

2. Supportive Care

- Blood Transfusion Support: Irradiated red blood cells or platelets will be transfused.

- Granulocyte Colony-Stimulating Factor: Starting on Day +6 post-transplant, 5-10 μg/kg/d (recipient weight) until myeloid engraftment.

- Routine Supportive Care: Supportive care deemed necessary by the investigator for patient recovery will be provided.

3. Management of Complications: Infections, GVHD, and other complications will be managed according to domestic/international guidelines.

Prohibited Medications: None
Follow-up Duration: - Enrollment Period (2 years) - Follow-up Period (1 year)
Dropout and Withdrawal Criteria: Subjects may withdraw from the study at their own request.
Exclusion Criteria:Not applicable.
Evaluation Endpoints: Primary Endpoint: Event-free survival (EFS) at 12 months post-transplant. Secondary Endpoints: 1. Neutrophil cumulative engraftment rate at 28 days post-transplant, and time to neutrophil engraftment. 2. Platelet cumulative engraftment rate at 28 days post-transplant, and time to platelet engraftment. 3. Incidence of Grade II-IV acute GVHD post-transplant. 4. Incidence of chronic GVHD post-transplant. 5. Survival rate. 6. Endocrine function (thyroid, gonads).
Statistical Methods Continuous variables will be compared using Student's t-test or Mann-Whitney U test. Categorical variables will be compared using Chi-square test or Fisher's exact test. Neutrophil cumulative engraftment rate, platelet cumulative engraftment rate, acute GVHD cumulative incidence, and chronic GVHD cumulative incidence will be analyzed using Gray's test with competing risks, considering death as a competing event for neutrophil and platelet cumulative engraftment rates, and death and primary/secondary graft failure as competing events for acute and chronic GVHD cumulative incidences. Overall survival (OS) and event-free survival (EFS) will be calculated using the Kaplan-Meier method, with group comparisons performed using the Log-rank test. Statistical analysis will be conducted using R4.2.2 software, with $P<0.05$ considered statistically significant.

2. Research Background

Refractory/relapsed aplastic anemia (AA) in adults remains a clinical challenge that is frequently encountered and urgently needs to be resolved. Refractory AA is ineffective to the standard first-line treatment regimen[1][2], which is based on antithymocyte globulin (ATG) combined with cyclosporine A (CsA) as the standard immunosuppressive therapy (IST), accounting for approximately 30%-40% of all AA patients. It has been reported that the 5-year mortality rate of refractory severe aplastic anemia (r-SAA) exceeds 40% after diagnosis[3]. Brosdky et al. reported that the 10-year event-free survival (EFS) of SAA treated with high-dose cyclophosphamide-based IST is only 28%. The efficacy is not satisfactory. Thrombopoietin receptor agonists such as eltrombopag, as an important treatment for r-SAA, have a hematologic response rate of only 41.8% with monotherapy for 52 weeks, with an overall low response rate and unsatisfactory quality of response. The 24-week complete blood count response is only 16.4%, and the 52-week response is only 21.8%. Clonal hematopoiesis is also a long-term concern for IST. Multiple centers have reported that the early efficacy of IST combined with thrombopoietin receptor agonists for treating SAA has improved, but the long-term overall efficacy has not substantially exceeded that of IST alone. The bone marrow failure of non-severe aplastic anemia (NSAA) is less severe than that of SAA, and the main treatment methods are IST $\pm$ thrombopoietin receptor agonists or combined with androgens, similar to SAA, with similar long-term efficacy challenges. Therefore, the development of new and effective treatment methods is an urgent clinical need.

Refractory AA is ineffective to IST, possibly mainly due to the insufficient number or functional abnormalities of residual hematopoietic stem/progenitor cells in patients. Therefore, salvage treatment should prioritize hematopoietic stem cell transplantation (HSCT) to supplement normal hematopoietic stem cells and rebuild hematopoiesis. According to domestic and international guidelines for the diagnosis and treatment of adult AA[1][4], unrelated donor allogeneic hematopoietic stem cell transplantation or haploidentical hematopoietic stem cell transplantation are the main salvage treatment methods for refractory SAA, with hematopoietic stem cells mainly derived from peripheral blood or bone marrow. However, the availability of suitable donors is one of the main difficulties for unrelated donor allogeneic hematopoietic stem cell transplantation or haploidentical hematopoietic stem cell transplantation. Many AA patients cannot receive early treatment due to the lack of suitable hematopoietic stem cell donors and have to wait for a long time. Unrelated cord blood is an ideal source of hematopoietic stem cells due to its easy availability, low immunogenicity, and low incidence of chronic graft-versus-host disease (cGVHD) after transplantation. However, Delatour et al.[5] reported that the 3-year overall survival (OS) of patients with refractory AA treated with cord blood transplantation is only 38% $\pm$ 6%, with unsatisfactory efficacy. Nevertheless, with the optimization of conditioning regimens, the popularization of high-resolution HLA typing, and the continuous improvement of supportive care, the efficacy of unrelated cord blood transplantation (UCBT) is expected to improve.

The optimization of the conditioning regimen for UCBT is a crucial factor in determining patient outcomes. Hiramoto et al.[6] reported that patients received conditioning regimens of total body

irradiation (TBI) (⩽ 4Gy) combined with fludarabine/melphalan (Flu/Mel) or fludarabine/cyclophosphamide (Flu/Cy). The results showed that a radiation dose of 4 Gy is an independent influencing factor for neutrophil engraftment. Compared with the Flu/Cy group, a higher proportion of patients in the Flu/Mel group received 4 Gy (96% vs. 68%, $p<0.01$), but there was no difference in engraftment success rates (77.8% vs. 72%). Compared with TBI, TMI has many advantages[7]: (1) TMI clears residual T cells in the recipient's body, optimizes spatial competition, and better prepares the bone marrow niche for cord blood stem cells; (2) It can more accurately target the bone marrow region; (3) It reduces toxicity to other organs and can better protect the patient's organ function; (4) It reduces the risk of long-term tumors; (5) It lowers the radiation dose: the TMI dose is 4-6 Gy, while the TBI dose reaches 12 Gy; (6) TMI is applicable to a wider range of people and is more tolerable for elderly/weak patients. Therefore, TMI has potential advantages in the conditioning regimen for refractory/relapsed severe aplastic anemia.

Therefore, we have designed and proposed for the first time the Phase II clinical study titled "A Phase II Study on the Efficacy and Safety of Umbilical Cord Blood Transplantation (UCBT) with a Total Marrow Irradiation (TMI)-Based Conditioning Regimen for the Treatment of Refractory/Relapsed Aplastic Anemia (AA) in Adults." This study employs a TMI (4 Gy)/Flu/Mel conditioning regimen and single-unit cord blood stem cell transplantation to treat adult refractory/relapsed AA, aiming to evaluate the efficacy and safety of this regimen.

3. Study Objectives

Primary Objective:
- Event-free survival (EFS) at 12 months post-transplant.

Secondary Objectives:
1) Neutrophil cumulative engraftment rate and time to engraftment at 28 days post-transplant.
2) Platelet cumulative engraftment rate and time to engraftment at 28 days post-transplant.
3) Incidence of Grade II-IV acute GVHD post-transplant.
4) Incidence of chronic GVHD post-transplant.
5) Survival rate at 12 months post-transplant.
6) Endocrine function (thyroid, gonads) at 12 months post-transplant.

4. Study Design

A total of 11 adult patients with refractory/relapsed aplastic anemia (AA) will be treated with a TMI-based conditioning regimen followed by single-unit unrelated cord blood transplantation (UCBT) to evaluate the efficacy and safety of this regimen.

5. Study Population

Adults with refractory/relapsed aplastic anemia.

5.1 Inclusion Criteria

Participants eligible for this study must meet all of the following criteria:

1) Age ≥18 years and <50 years, regardless of gender.

2) Diagnosis of aplastic anemia according to the guidelines of the British Committee for Standards in Haematology; hypocellular bone marrow without infiltration of abnormal cells or bone marrow fibrosis; peripheral blood must meet at least two of the following three criteria: absolute neutrophil count (ANC) <1.5 × 10^9/L; platelet count <50 × 10^9/L; hemoglobin <100 g/L.

- Diagnostic Criteria for Severe Aplastic Anemia (SAA):

  - Peripheral Blood Criteria: Meet at least two of the following three criteria: ANC <0.5 × 10^9/L; reticulocyte count <20 × 10^9/L; platelet count <20 × 10^9/L.

  - Bone Marrow Criteria: Bone marrow cellularity <25% of normal; if ≥ 25% but <50% of normal, then the proportion of residual hematopoietic cells should be <30%. Bone marrow aspiration shows hypocellular or severely hypocellular marrow with a marked reduction in hematopoietic cells and an increase in non-hematopoietic cells such as lymphocytes and reticular cells.

  - Other: Exclude other diseases causing pancytopenia, such as myelodysplastic syndrome (MDS), paroxysmal nocturnal hemoglobinuria (PNH), and congenital bone marrow failure syndromes.

If ANC <0.2 × 10^9/L, the diagnosis is very severe aplastic anemia (VSAA); those who do not meet the SAA criteria are classified as non-severe aplastic anemia (NSAA).

3) Meet the criteria for refractory aplastic anemia:

  - For SAA: Ineffective after 6 months of first-line ATG + CSA treatment.

  - For NSAA: Meet one of the following criteria: (1) Persistent transfusion dependence in two blood cell lines; (2) Ineffective after 12 months of immunosuppressive therapy with cyclosporine or other agents, or ineffective after 6 months of treatment with androgens and/or TPO receptor agonists, or progression to SAA.

Meet the criteria for relapsed aplastic anemia: Initially effective to first-line immunosuppressive therapy, but with subsequent pancytopenia reaching SAA criteria after tapering or discontinuation of immunosuppressive therapy.

4) Karnofsky Performance Status ≥ 60 points, ECOG score ≤ 2, Hematopoietic Cell Transplantation - Comorbidity Index (HCT-CI) ≤2.

5) No HLA-identical sibling donor or no HLA-identical unrelated donor available.

6) Understand the study procedures and voluntarily provide written informed consent.

5.2 Exclusion Criteria

Participants with any of the following conditions are not eligible for this study:

1) Other causes of pancytopenia and hypocellular bone marrow diseases (including PNH, etc.).

2) Use of medium-to-high doses of cyclophosphamide (≥20 mg/kg/day) for immunosuppressive therapy within 3 months prior to enrollment.

3) Previous history of hematopoietic stem cell transplantation.

4) Known or suspected contraindications or allergies to fludarabine, melphalan, or other drugs.

5) Uncontrolled bleeding and/or infection after standard treatment prior to screening.
6) Active viral hepatitis (hepatitis B, hepatitis C, etc.), HIV infection, or syphilis at screening or prior to treatment.
7) Creatinine clearance rate <60 ml/min or serum creatinine >140 μ mol/L prior to treatment.
8) Pregnant or breastfeeding women.
9) Participation in another clinical trial within 3 months prior to enrollment.
10) Any other condition that the investigator deems may prevent the participant from completing the study or pose significant risks to the participant.

5.3 Withdrawal and Discontinuation Criteria

Participants may withdraw from the study at any time at their own request.

5.4 Exclusion Criteria

Not applicable.

6. Study Regimen: Total Marrow Irradiation (TMI)-Based Conditioning Regimen

6.1 Radiotherapy Simulation and Positioning:

1. Environmental and Surface Disinfection: 12 hours and 2 hours before radiotherapy simulation, the molding room and CT simulation room will be disinfected with a UV disinfection machine for 30 minutes. The surface of the positioning mold, the operating table, and the treatment bed of the simulation CT machine will be wiped three times with 75% ethanol.
2. Personal Protective Equipment and Operating Standards: Before simulation, therapists must wear isolation gowns, gloves, masks, caps, and shoe covers, and strictly follow aseptic operating procedures.
3. Patient Education: Patients will be thoroughly educated on the specific procedures and purposes of molding, simulation, and treatment.
4. Patient Positioning and Mold Fabrication: Patients will be positioned using a head-body integrated board, vacuum cushion, head and neck thermoplastic mask, head and neck positioning pad, and body thermoplastic mask. Marking lines will be drawn on the patient's hands, feet, and the boundaries with the vacuum cushion, and the upper and lower limits of the mold will be clearly marked. After shaping, 5-6 sets of positioning coordinates will be marked on the head, body, and legs from top to bottom under the guidance of a laser light, and a lead wire will be horizontally marked on the upper part of the thigh.
5. Simulation CT Scan: The scan will be performed with an appropriate field of view (FOV) in free-breathing state, with a slice thickness of 5 mm. The scanning range will be set from 5 cm above the top of the skull to 5 cm below the toes. If the patient's height exceeds 180 cm, the thigh lead wire marking will be used for segmented scanning: the upper segment will be scanned from 5 cm above the top of the skull to 5 cm below the lead wire; for the lower segment, the patient will be repositioned with feet forward, and the scanning range will be set from 5 cm above the toes to 5 cm below the lead wire. After the CT scan is completed, the images will be

uploaded to the Eclipse workstation.

6.2 Radiotherapy Target Volume Delineation:

1. The target volume will be delineated on the Eclipse radiotherapy physician workstation, including the major lymphatic chains and the entire bone marrow of the body. The major lymphatic chains will include the internal jugular lymph nodes, supraclavicular lymph nodes, axillary lymph nodes, mediastinal lymph nodes, para-aortic lymph nodes, iliac vessel lymph nodes, inguinal lymph nodes, and spleen. The entire bone marrow will be represented by the entire skeleton and will be automatically delineated using a system tool, excluding the maxilla and mandible to reduce oral mucosal reactions. Additionally, since the ribs and spleen are significantly affected by respiratory motion, the ribs will be delineated together with the chest wall according to the motion range on the CT images at the end of inspiration and expiration, and the spleen will be delineated according to its motion range by fusing the CT images at the end of inspiration and expiration.
2. The major lymphatic chains and the entire skeleton will be divided into different regions and named accordingly.
3. Organs at risk will be delineated, including the lens, oral cavity, parotid glands, thyroid gland, breasts (female), lungs, heart, liver, gastrointestinal tract, kidneys, spinal cord, ovaries (female), and testes (male).

6.3 Radiotherapy Prescription Dose: According to literature reports and clinical experience of the radiotherapy center, 6MV-X rays will be used for single-fraction 4 Gy irradiation.

6.4 Radiotherapy Plan Design:

Based on the maximum field width of the multileaf collimator built into the HALCYON linear accelerator at our hospital, 12-15 treatment centers will be set according to the patient's height for segmented irradiation. The body will be planned using volumetric modulated arc therapy (VMAT) technology, with 2-4 arcs per treatment center; the lower limbs will be planned using intensity-modulated radiation therapy (IMRT) technology, with 3-5 fields per treatment center, resulting in a total of 6-8 radiotherapy plans. The prescription dose and dose limits for organs at risk will be entered into the treatment planning system for inverse planning optimization. The plan will be output after meeting the planning requirements.

6.5 Radiotherapy Plan Review:

The volume receiving 100% of the prescription dose (V4) should be no less than 85% of the planning target volume (PTV), i.e., V4>85%. The maximum dose (Dmax) within the target area should be less than 4.4 Gy, with the volume receiving the maximum dose not exceeding 2% of the PTV volume.

Dose limits for organs at risk: The mean dose (Dmean) to the lens, lungs, and kidneys should be less than 50%-60% of the prescription dose. The maximum dose (Dmax) to the ovaries and testes

should be less than 2 Gy.

6.6 Radiotherapy Plan Decomposition and Verification:

1. Plan decomposition.
2. Dose verification: After plan decomposition, the gamma pass rate should be ≥95% (3 mm/3%) using the Halcyon Portal Dosimetry technique and the Arc Check phantom for different sub-plans.
3. Position verification: After qualified plan dose verification, a three-dimensional position simulation verification will be performed in the Halcyon accelerator treatment room for head-first and feet-first treatment simulations.

6.7 Radiotherapy Implementation:

1. Environmental and Surface Disinfection: 12 hours and 2 hours before radiotherapy, the accelerator treatment room will be disinfected with a UV disinfection machine for 30 minutes. The surface of the positioning mold and the linear accelerator treatment bed will be wiped three times with 75% ethanol.
2. Personal Protective Equipment and Operating Standards: Before treatment, therapists must wear isolation gowns, gloves, masks, caps, and shoe covers, and strictly follow aseptic operating procedures to ensure a safe and standardized treatment environment and process.
3. After bathing, patients will enter the accelerator treatment room wearing disinfected clean clothing. Therapists will carefully verify the patient's identity and treatment information. Given the patient's weakened condition, assistance will be provided to help the patient onto the treatment bed safely. The patient will be positioned head-first, and the positioning marks on the body will be carefully aligned, especially for areas with significant movement. Once the position is confirmed, the mold will be secured.
4. Before starting irradiation, the cone beam CT (CBCT) built into the linear accelerator will be used to verify the position based on bony structures, ensuring that the positioning error is within 5 mm. The treatment of the upper body will then commence. During treatment, therapists must accurately record the couch values and precisely calculate the X, Y, and Z-axis coordinates for the next field to ensure seamless between fields and prevent target overlap leading to cumulative dose or gaps causing insufficient dose.
5. During radiotherapy implementation, a multi-channel dosimeter system will be used to monitor key points on the patient, assessing dose uniformity in areas such as the head and neck, lungs, and pelvis.
6. According to the radiotherapy plan, after the upper part of the body is treated, the patient will be assisted off the couch for a brief rest, during which they can hydrate. Technicians will then switch the direction of the body board, and the patient will be assisted back onto the couch in a feet-first position. The positioning marks on the body will be aligned again, especially for joints with significant movement. Once confirmed, the mold will be secured, and treatment of the lower body will commence. The treatment will continue until completion, with real-time video monitoring throughout the process. For patients with a target area less than 100 cm, continuous segmented irradiation can be performed without repositioning. If the patient's target area

exceeds 100 cm, the patient will need to be repositioned with feet forward after the upper part of the body is irradiated, and the lower part will be treated using the thigh lead wire marking as the boundary.

7. After treatment is completed, the dose information will be carefully verified for accuracy. Once confirmed, the patient will be assisted off the couch safely, dressed in isolation gown, gloves, and mask, and escorted out of the treatment room. The patient will then return to the ward wearing disinfected clean clothing.

7. Medication Plan

1. Conditioning Regimen:
Flu 30 mg/m^2 × 5 days (Days -6, -5, -4, -3, -2);
TMI 4 Gy qd (Day -2);
Mel 100 mg/m^2 qd (Day -1).

2. Single-Unit Cord Blood:
- Cord Blood Source: Cord blood units will be sourced from the Chinese Public Cord Blood Hematopoietic Stem Cell Bank.
- Cord Blood Quality Requirements:
  - Maternal Conditions: Age typically 18-35 years (some institutions extend to 40 years). No chronic diseases (such as diabetes, hypertension), no history of infections or medication use during pregnancy. No history of multiple miscarriages or fetal malformations.
  - Cord Blood Quality Standards:
    - Total nucleated cell (TNC) count: Based on domestic single-unit UCBT clinical transplant data, it is recommended that the TNC count be ≥2.0-4.0×10^7/kg (recipient's body weight) before freezing, and ≥ 1.5 × 10^7/kg (recipient's body weight) after thawing. If TNC is insufficient, prioritize cord blood with a higher CD34+ cell count, as CD34+ cell count is more important than TNC.
    - CD34+ cell count: CD34+ cell count is a factor in accelerating granulocyte engraftment. However, there is no standard for the minimum CD34+ cell count required for engraftment. A retrospective study of 620 single-unit UCBT cases in a Chinese center confirmed that when the CD34+ cell count is ≥0.83×10^5/kg and HLA mismatch is ≤3/10, good engraftment and overall survival (OS) can be achieved. It is recommended to select cord blood with a CD34+ cell count ≥ 1.2 × 10^5/kg (recipient's body weight) before freezing, and ≥ 1.0 × 10^5/kg (recipient's body weight) after thawing, with CD34+ cell viability >85%. For urgent single-unit UCBT cases, cord blood with a CD34+ cell count ≥0.83×10^5/kg (recipient's body weight) can be selected for transplantation.
    - Sterility: Culture negative, no maternal red blood cell contamination (detected by hemoglobin F ratio).
  - HLA Matching Requirements:
    - Minimum matching degree: The HLA compatibility between the cord blood and the patient should be ≥4/6, 5/8, 6/10.
    - Preferred matching: 5-6/6 match, or HLA-C match (required by some centers).
    - DSA: Within 2 months prior to transplantation, patients will undergo HLA antibody testing

to select DSA-negative cord blood.

- Exclusion Criteria:
  - Maternal infectious disease positivity (e.g., HIV, active hepatitis B).
  - Fetal chromosomal abnormalities or genetic disease risks.
  - Insufficient cord blood collection volume or low cell viability.

8. Prohibited Medications: None

9. Concomitant Therapy:

1. Complication Prevention:
- VOD Prevention: Ursodeoxycholic acid 250 mg tid orally, starting with conditioning regimen; Prostaglandin E1 10 μg qd intravenous injection, stopping after 30 days; Low molecular weight heparin 100 IU/kg/day intravenous infusion, starting with conditioning regimen (discontinue if platelet count <20×10^9/L).
- CMV Prevention: For CMV-IgG positive patients, letermovir will be administered starting on Day +7 for 3 months, according to relevant guidelines.
- Herpes Virus Prevention: Acyclovir 10 mg/kg/day, divided into two doses per day, starting on Day +1 and continuing for 1 year.
- Fungal Prevention: For patients undergoing UCBT, antifungal prophylaxis will be initiated simultaneously with the conditioning regimen and continued for at least 3 months post-transplant. If acute or chronic GVHD or immunosuppressive therapy is present, the course will be extended until GVHD symptoms are controlled and immunosuppressive agents are tapered off. Triazole or echinocandin antifungal agents will be used for primary prophylaxis. For patients with a history of confirmed or clinically diagnosed invasive fungal disease (IFD), antifungal therapy will be continued for at least 3 months post-transplant, extending until GVHD symptoms are controlled and immunosuppressive agents are tapered off, following relevant guidelines.

2. Supportive Care:
- Transfusion Support: Irradiated red blood cells or platelets will be transfused as needed.
- Granulocyte Colony-Stimulating Factor (G-CSF): Starting on Day +6 post-transplant, 5-10 μg/kg/day (recipient's body weight) until myeloid engraftment.
- Routine Supportive Care: Any other supportive care deemed necessary by the investigator to aid in patient recovery.

3. Management of Complications: Infections, GVHD, and other complications will be managed according to domestic and international guidelines.

10. Follow-Up Schedule:

10.1 Enrollment Period (2 weeks): Baseline information including previous treatment history, medical history, and demographic data will be collected. Physical examination, vital signs, blood and urine tests, liver and kidney function tests, electrocardiogram (ECG), CT/MRI scans, clinical

manifestations, and ECOG performance status will be recorded. Informed consent for enrollment will be obtained.

10.2 Treatment Period (7 days): Patients will receive the conditioning regimen of TMI (4 Gy)/Flu/Mel followed by single-unit cord blood stem cell transplantation.

10.3 Follow-Up Period (12 months): Clinical manifestations, blood tests, biochemical parameters, and immune reconstitution will be monitored over the 12-month period post-transplant.

11. Evaluation Criteria:

11.1 Efficacy Evaluation:

1) Neutrophil engraftment: Defined as an absolute neutrophil count (ANC) >0.5 × 10^9/L for three consecutive days.
2) Platelet engraftment: Defined as a platelet count >20×10^9/L for at least 7 consecutive days without platelet transfusion support.
3) Overall survival (OS): Defined as the time from cord blood stem cell transplantation to death or last follow-up.
4) Primary graft failure: Defined as the absence of neutrophil engraftment within 42 days post-UCBT.
5) Secondary graft failure: Defined as the loss of donor cells after initial engraftment post-UCBT.
6) Event-free survival (EFS): Defined as the time from the start of UCBT to the occurrence of an event, including death, graft failure, lack of response, relapse, and clonal progression.

11.2 Safety Evaluation:

1) Acute GVHD: Diagnosis and severity grading will be based on reference [8].
2) Chronic GVHD: Diagnosis and severity grading will be based on reference [9].

12. Adverse Events and Serious Adverse Events

All adverse events occurring from the time of signing the informed consent form until the last follow-up visit will be recorded.

12.1 Adverse Events

12.1.1 Definition of Adverse Events

Adverse events are any unfavorable medical occurrences that appear after a subject's participation in a clinical study, which are not necessarily related to the investigational treatment. They include any new occurrences or worsening of existing conditions compared to the baseline, including abnormal results from laboratory tests or physical examinations. Adverse events include serious adverse events (SAEs), adverse events (AEs), and abnormal laboratory results.

12.1.2 Collection and Recording of Adverse Events

All adverse events occurring from the time of signing the informed consent form until the last follow-up visit will be recorded. Investigators will report all adverse events that are possibly related to the investigational treatment using concise medical terminology.

12.1.3 Severity Assessment of Adverse Events

The severity of adverse events will be assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0, which classifies events into five grades:

- Grade 1: Mild; asymptomatic or only clinically or diagnostically observed; no treatment required.
- Grade 2: Moderate; requiring minimal, local, or non-invasive treatment; limited to instrumental activities of daily living (IADL) appropriate for age.
- Grade 3: Severe or medically significant but not immediately life-threatening; resulting in hospitalization or prolongation of hospital stay; causing disability; limited to personal activities of daily living (ADL).
- Grade 4: Life-threatening; requiring urgent treatment.
- Grade 5: Death related to the adverse event (AE).

Instrumental Activities of Daily Living (IADL): Activities such as cooking, shopping, using the telephone, managing finances, etc.
Personal Activities of Daily Living (ADL): Activities such as bathing, dressing, eating, toileting, taking medications, etc., without being bedridden.

Note the distinction between the severity and intensity of adverse events. Intensity describes the degree of severity, but it does not necessarily equate to an SAE. For example, a headache may be intense but is not considered an SAE unless it meets the criteria for an SAE.

12.1.4 Assessment of Causality of Adverse Events

Investigators will assess the relationship between adverse events and the investigational treatment based on the following criteria:

Causality	Time Relationship	Consistency with Known Types of Adverse Reactions	Other Possible Causes of the Adverse Event
Definite	Yes	Yes	No
Probable	Yes	Yes/No	Uncertain
Possible	No	No	Uncertain
Unlikely	No	No	Yes
Unassessable	Insufficient data to evaluate		

Adverse events classified as "definite," "probable," "possible," and "unassessable" will be considered adverse reactions. Investigators must record adverse reactions using medical terminology.

12.2 Serious Adverse Events

12.2.1 Definition of Serious Adverse Events

A serious adverse event is defined as any of the following:

1) Death or life-threatening condition.
2) Hospitalization or prolongation of existing hospitalization.
3) Persistent or significant disability/incapacity.
4) Congenital anomaly/birth defect.
5) Medically significant condition that poses a threat to the patient's health and requires medical or surgical intervention to prevent one of the outcomes listed above.

The following hospitalizations are typically not considered serious adverse events:

1) Hospitalization due to disease progression in subjects who withdraw or discontinue the study.
2) Hospitalization for routine treatment or observation of the condition being studied, without worsening of symptoms.
3) Hospitalization that was planned prior to enrollment in the study, and pre-existing symptoms have not worsened.
4) Treatment in an emergency or outpatient setting that does not meet the definition of a serious adverse event and does not require hospitalization.
5) Pregnancy itself is not considered a serious adverse event, but it should be reported on the serious adverse event form or pregnancy record form, with follow-up and documentation of the outcome, including spontaneous abortion, elective termination, and details of childbirth, presence of congenital abnormalities, and birth defects.

12.2.2 Reporting of Serious Adverse Events

If a subject experiences a Grade 5 serious adverse event during the study (from the time of signing the informed consent form until the last follow-up visit) that is possibly related to the investigational treatment, the investigator must complete a serious adverse event report form and submit it to the ethics committee of the research center within 24 hours of becoming aware of the event. The investigator must sign and date the report.

Contact Information for the Ethics Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Hematology:

- Contact Person: Liu Xue'ou
- Phone: 022-23909095
- Email: ec@ihcams.ac.cn
- Address: No. 288 Nanjing Road, Heping District, Tianjin, China

13. Data Management

Data will be collected using case report forms (CRFs). A designated person at the research center will enter the data from the CRFs into the database and verify the consistency between the database and the CRF data. Data administrators will review the data in the database according to the clinical research protocol. If any questions arise, a query list will be completed, and the investigator will address the issues listed. The query list will be properly stored. Data administrators will be responsible for exporting the data from the database for statistical analysis.

14. Statistical Analysis

14.1 Statistical Analysis Sets

- Full Analysis Set (FAS):Includes data from all subjects. Used for reporting compliance and all baseline characteristic analyses. Efficacy analysis will be based on the FAS.
- Safety Analysis Set (SS): Includes all subjects who have received treatment and have safety records. Safety analysis will be based on the SS.

14.2 Statistical Analysis Methods

Continuous variables will be compared using Student’s t-test or Mann-Whitney U test. Categorical variables will be compared using Chi-square test or Fisher’s exact test. Neutrophil cumulative engraftment rate, platelet cumulative engraftment rate, cumulative incidence of acute GVHD, and chronic GVHD will be analyzed using Gray’s test with competing risks, considering death as a competing event for neutrophil and platelet engraftment rates, and death, primary graft failure as competing events for acute and chronic GVHD incidence. Overall survival (OS) and event-free survival (EFS) will be calculated using the Kaplan-Meier method, with group comparisons performed using the Log-rank test. Statistical analysis will be conducted using R 4.2.2 software, with $p<0.05$ considered statistically significant.

14.3 Sample Size Calculation

This trial employs a single-arm Phase II study design with the primary endpoint being the event-free survival (EFS) at 12 months post-transplant. Based on historical studies, the 12-month complete response (CR) rate is approximately 24% [10]. The expected 12-month EFS for refractory/relapsed aplastic anemia is set at 20%, while the anticipated EFS for this trial is 60%. With a significance level (α) of 0.05 and a power (1- β) of 80%, and considering an enrollment period of 2 years and a follow-up period of 1 year, the calculated sample size is 10 subjects. Accounting for a 10% dropout rate, the final sample size is set at 11.

15. Ethical Review

15.1 Ethical Requirements

The implementation of this study will adhere to the current Declaration of Helsinki (2013), relevant regulations, and the review opinions of the ethics committee. Prior to the commencement of the study, investigators must obtain written approval from the relevant regulatory authorities for the study protocol, informed consent form, subject recruitment procedures, and any other written materials provided to subjects. During the course of the study, any amendments to the study protocol, informed consent form, or other materials must also be submitted for written approval by the relevant regulatory authorities.

15.2 Informed Consent

The investigator or their designated representative will be responsible for explaining to each subject (as applicable), the parents/legal guardians of the subject, or a witness, the background of the study, the pharmacological characteristics of the investigational medical technology, the study protocol, as well as the potential benefits and risks associated with participation. Written informed consent must be obtained from the subject (as applicable), the parents/legal guardians, and the investigator prior to the subject's enrollment in the study (before any screening procedures). The final version of the informed consent form must include the following elements: study purpose, study procedures, obligations of the subject, foreseeable benefits to the subject, foreseeable risks and inconveniences, compensation for any study-related injuries, data access and confidentiality of subject information, etc. The informed consent form must be approved by the relevant regulatory authorities and written in a language that the subject (as applicable) or the parents/legal guardians can understand. Both the subject (as applicable), the parents/legal guardians, and the investigator or their representative must sign and date the informed consent form. One copy of the signed informed consent form should be retained by the investigator and another by the subject. If any significant new information related to the study is discovered, the informed consent form must be amended in writing, submitted for approval by the relevant regulatory authorities, and re-consent obtained from the subject.

For subjects who are unable to participate in the informed consent process, written informed consent must be obtained from the subject's parents/legal guardians, and all study procedures must be explained to them.

15.3 Confidentiality of Subjects

Investigators are responsible for maintaining the anonymity of subjects. Case report forms or other documents should identify subjects using uppercase letters, numbers, and/or codes, rather than their names. Investigators must maintain a secure record linking subject codes, names, and addresses. Strict confidentiality must be maintained for any documents that reveal subject identity.

16. Study Management

16.1 Training

Prior to the commencement of the clinical study, investigators must undergo training on the study protocol, thoroughly familiarize themselves with its content, and standardize the methods of recording and criteria for judgment. The protocol must be strictly followed.

16.2 Quality Control and Assurance

All observed results and abnormal findings during the clinical study must be promptly verified and recorded to ensure the reliability of the data. Investigators will enter the required information into the case report forms, which will be verified for completeness and accuracy by monitors.

17. Anticipated Study Progress and Completion Date

The study schedule is tentatively planned as follows: June 2025 to June 2028.
- Enrollment of the first subject: June 2025.
- Completion of the last subject's follow-up: December 2027.
- Data management, statistical analysis, and clinical report: June 2028.

18. Confidentiality and Publication of Study Results

Investigators must maintain confidentiality regarding all information and data related to this study. Without the consent of the research institution, no related study results or data may be cited or published.

19. References

1. Kulasekararaj A, Cavenagh J, Dokal I, et al. Guidelines for the diagnosis and management of adult aplastic anaemia: A British Society for Haematology Guideline. Br J Haematol 2024;204:784-804.
2. Peffault de Latour R, Kulasekararaj A, Iacobelli S, et al. Eltrombopag Added to Immunosuppression in Severe Aplastic Anemia. N Engl J Med 2022;386:11-23.
3. Valdez JM, Scheinberg P, Nunez O, Wu CO, Young NS, Walsh TJ. Decreased infection-related mortality and improved survival in severe aplastic anemia in the past two decades. Clin Infect Dis 2011;52:726-35.
4. Red Blood Cell Disease Group CSoHCMA. [Guidelines for the diagnosis and management of aplastic anemia in China (2022)]. Zhonghua xueyexue zazhi 2022;43:881-8.
5. Peffault de Latour R, Purtill D, Ruggeri A, et al. Influence of nucleated cell dose on overall survival of unrelated cord blood transplantation for patients with severe acquired aplastic anemia: a study by eurocord and the aplastic anemia working party of the European group for blood and marrow transplantation. Biol Blood Marrow Transplant 2011;17:78-85.
6. Hiramoto N, Yamazaki H, Nakamura Y, et al. Total body irradiation-containing conditioning regimens without antithymocyte globulin in adults with aplastic anemia undergoing umbilical cord blood transplantation. Annals of hematology 2022;101:165-75.
7. Kurtzberg J, Troy JD, Page KM, et al. Unrelated Donor Cord Blood Transplantation in Children:

Lessons Learned Over 3 Decades. Stem Cells Transl Med 2023;12:26-38.
8. Harris AC, Young R, Devine S, et al. International, Multicenter Standardization of Acute Graft-versus-Host Disease Clinical Data Collection: A Report from the Mount Sinai Acute GVHD International Consortium. Biol Blood Marrow Transplant 2016;22:4-10.
9. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. Biol Blood Marrow Transplant 2015;21:389-401 e1.
10. Olnes MJ, Scheinberg P, Calvo KR, et al. Eltrombopag and improved hematopoiesis in refractory aplastic anemia. N Engl J Med 2012;367:11-9.

20. Appendices

1. Investigator-Initiated Clinical Study Project Information Form (Version 1.8, May 28, 2025)
2. Clinical Study Protocol (Version 1.8, May 28, 2025)
3. Informed Consent Form (Version 1.8, May 28, 2025)
4. IIT Study Agreement: Not applicable.
5. IIT Study Authorization to Commence Form (Version 1.8, May 28, 2025)
6. Study Closure Review Application Form (Version 1.8, May 28, 2025)
7. Hematology Institute Research Agreement Template - CRC Tripartite Agreement (2021) - No cooperating party applicable.