Study Protocol

Project Title: Validation of an intensified treatment strategy for disseminated MAC infection: a four-drug regimen with the addition of a fluoroquinolone (levofloxacin or moxifloxacin) to the standard triple therapy — a multicenter, randomized, controlled clinical trial
Sponsor: Shanghai Public Health Clinical Center
Principal Investigator: Liu Li
Version No.: 2.1
Version Date: April 11, 2026

Protocol Signature Confirmation:

Compliance Statement

This study shall comply with the provisions of the Good Clinical Practice for Drug Trials, the Management Measures for Investigator-Initiated Clinical Research Conducted by Medical and Health Institutions, and the Declaration of Helsinki. We hereby commit to conducting this study in accordance with this protocol. All participants must be trained, and the study may only be initiated after written approval from the ethics committee and written informed consent from the study participants has been obtained. Any protocol amendment must be reapproved before implementation.

1. Study Background and Rationale

Disseminated infection with Mycobacterium avium complex (MAC) is one of the major opportunistic infections in patients with advanced HIV infection. It occurs in populations with severe immunosuppression, commonly those with CD4 counts <50 cells/μL, and in those with unsuppressed viremia or “late diagnosis, late treatment.” With the advent of highly active antiretroviral therapy (ART), the overall incidence of disseminated MAC (DMAC) has declined substantially. However, in the real-world context where a considerable proportion of patients are still hospitalized late, interrupt or fail ART, or have multiple concurrent opportunistic infections, DMAC remains a clinically significant burden. Based on laboratory surveillance data from Oregon, USA (2007–2012), disseminated nontuberculous mycobacterial disease, mainly MAC, in people with HIV was concentrated in those with CD4 counts <50 cells/μL, indicating that the risk remains extremely high in severely immunosuppressed populations. At the same time, retrospective cohort studies have shown that although ART can reduce the occurrence of DMAC, new DMAC events still occur in patients with persistently very low CD4 counts. In addition, the benefit of MAC prophylaxis has not been entirely consistent across studies, reflecting the complex relationship between the disease and the pace of immune reconstitution, prior exposure, and the spectrum of co-infections.

From a pathogenesis perspective, MAC is an environmental opportunistic pathogen that may colonize the respiratory or gastrointestinal tract. “Disseminated disease” usually indicates that the pathogen has breached the mucosal barrier and entered the reticuloendothelial system and bloodstream, involving the bone marrow, liver, spleen, intestines, and lymph nodes. Clinically, it often presents with unexplained fever, night sweats, weight loss, diarrhea/abdominal pain, anemia, and elevated alkaline phosphatase. Because these manifestations overlap with those of other opportunistic infections, such as PCP, tuberculosis, and cryptococcosis, early diagnosis and timely, standardized treatment are challenging. Some guidelines also emphasize distinguishing “colonization/isolation” from “true disseminated disease” and recommend obtaining blood cultures, bone marrow specimens, or sterile-site specimens as soon as possible when suspicion is high in order to establish evidence for diagnosis. In the early phase after ART initiation, DMAC may also present as immune reconstitution inflammatory syndrome (IRIS) or become “unmasked.” The severity of clinical manifestations varies widely, and abdominal/deep organ involvement often indicates a greater burden of disability and hospitalization. Therefore, DMAC treatment strategies should not only aim for long-term cure, but also focus on early pathogen clearance, complications—especially IRIS—and the interactions and tolerability of anti-infective drugs with ART.

Regarding the evidence base for standard treatment, multiple authoritative guidelines regard a macrolide (clarithromycin or azithromycin) + ethambutol (EMB) + an optional rifabutin/rifampin as the core first-line framework for DMAC and clearly discourage macrolide monotherapy in order to reduce the risks of resistance and treatment failure. The 2024 BHIVA guideline recommends that the preferred regimen for DMAC include a macrolide and ethambutol, with rifabutin or rifampin as part of the first-line combination, while also emphasizing drug interactions and dose adjustment. This recommendation is based on several randomized studies. For example, Benson et al. enrolled patients with MAC bacteremia in a randomized trial comparing clarithromycin + ethambutol (C+E), clarithromycin + rifabutin (C+R), and clarithromycin + ethambutol + rifabutin (C+E+R). Although the primary endpoint did not differ substantially among the regimens, the triple-drug combination demonstrated advantages in survival and in important outcomes such as relapse/resistance, supporting the use of triple therapy in patients with severe disease, poor immune reconstitution, or prolonged treatment needs in order to reduce relapse and emergence of resistance. In addition, a 1996 NEJM study comparing rifabutin + ethambutol + clarithromycin with rifampin + ethambutol + clofazimine + ciprofloxacin showed that the former achieved faster and more frequent blood culture clearance and better survival, further strengthening the evidence base for using a macrolide backbone combined with ethambutol and a rifamycin. Treatment manuals for opportunistic infections in Ontario/BC and other regions also summarize that in previous studies, adding rifabutin may help protect against the emergence of macrolide resistance, and suggest that triple therapy may be preferable in patients with profound CD4 depletion, high organism burden, or ineffective ART.

However, the “standard triple therapy” for DMAC still faces multiple limitations in real-world practice. First, DMAC is often associated with a high pathogen burden and systemic involvement, and early blood culture or lesion culture conversion may be suboptimal, resulting in persistent fever, anemia, malnutrition, and hospitalization needs, with early mortality still present. Second, DMAC often involves the gastrointestinal tract, where drug malabsorption may occur; combined with polypharmacy due to co-infections, adequate exposure to effective drugs is difficult to ensure. The BHIVA guideline specifically notes that gastrointestinal involvement in DMAC may lead to absorption problems and that interactions between antimycobacterial drugs and ART or other medications must be systematically assessed and monitored. Third, adverse effects and drug interactions may limit completion of therapy. For example, rifabutin has significant interactions with some ART regimens and requires dose adjustment; clarithromycin and rifabutin have bidirectional interactions that may increase adverse-event risks and affect efficacy, making azithromycin more practical in some populations. Fourth, as ART strategies emphasize rapid initiation, the timing of DMAC treatment relative to ART initiation must be balanced. Delayed ART is unfavorable for immune recovery, while overly early ART may increase IRIS and clinical instability. In practice, ART is usually initiated within a short window after anti-mycobacterial treatment, with close monitoring. Given these complexities, “how to improve early pathogen clearance without significantly increasing toxicity and interactions” is a clinically meaningful research question.

Against this background, an “intensified phase four-drug” strategy has a clear theoretical and practical rationale. On the one hand, the treatment goal for DMAC is not only final cure and relapse prevention over a long treatment course, but also rapid reduction of organism burden, relief of systemic inflammation, improvement of clinical status, and shortening of the hospitalization/complication window. On the other hand, existing evidence suggests that differences among drug combinations in DMAC can affect blood culture conversion speed and survival, providing an evidence-based direction for exploring a better intensified regimen. Therefore, the primary endpoint of this study is culture conversion by Day 28, aiming to capture the effect of intensified therapy on early bactericidal activity and clearance speed. This time point is close to the hospitalization and initial treatment decision window, facilitates timely clinical assessment of efficacy, supports adherence and interaction management, and provides a basis for subsequent maintenance therapy and ART integration.

Regarding the rationale for adding a fluoroquinolone (levofloxacin or moxifloxacin) on top of triple therapy: in addition to the standard “triple regimen” (macrolide + ethambutol + rifabutin/rifampin), guidelines and real-world studies suggest that a fourth drug may be considered in some severe/high-burden DMAC cases to accelerate microbiological response and improve early outcomes. A previous retrospective cohort found that adding a fluoroquinolone (such as ciprofloxacin) to clarithromycin + ethambutol + a rifamycin was associated with better survival, suggesting potential synergistic benefit. The in vitro activity of fluoroquinolones against mycobacteria and their pharmacokinetic characteristics (good tissue distribution) provide a pharmacologic

basis for adding them during the intensified phase. However, the optimal agent and the population most likely to benefit in HIV-associated DMAC remain unsupported by prospective randomized evidence. Therefore, in this study, a fluoroquinolone will be added to standard triple therapy during the intensified phase (the first 8 weeks), with the investigator allowed to choose either levofloxacin or moxifloxacin at randomization, so as to accommodate availability across centers and individual safety considerations. In general, moxifloxacin 400 mg once daily usually does not require renal dose adjustment, but carries a relatively greater risk of QT prolongation; levofloxacin has a relatively lower QT risk but requires dose adjustment according to renal function. To reduce selection bias and crossover intervention, investigators are required to determine the specific fluoroquinolone at randomization and, in principle, not switch it during the intensified phase unless adverse reactions or clear contraindications occur.

Of course, the potential risks of fluoroquinolones are also a part of the study that must be simultaneously validated and strictly controlled. Systemic fluoroquinolones can cause tendinitis/tendon rupture, peripheral neuropathy, central nervous system adverse effects, and dysglycemia, some of which may be severe, persistent, or even irreversible. Regulatory agencies have repeatedly issued safety warnings and recommended cautious use only when benefits are clear. In terms of cardiac safety, fluoroquinolones may prolong the QT interval and increase the risk of torsades de pointes. Moxifloxacin is generally considered to have a more pronounced QT-prolonging effect, whereas levofloxacin is relatively weaker but not risk-free. Based on these risks, this study will systematically assess arrhythmia/QTc and electrolytes before enrollment, perform stratified ECG monitoring during the intensified phase, and establish explicit stopping/adjustment thresholds and management procedures to maximize participant safety.

Therefore, while maintaining microbiological outcomes as important secondary endpoints, this study sets Day 28 clinical symptom relief as the primary endpoint to better reflect early patient benefit and clinically perceptible outcomes. Day 28 is a key decision window for hospitalization and initial intensified treatment. Symptom relief—including defervescence, improvement in systemic symptoms, weight gain and improved functional status—can comprehensively reflect the combined effects of pathogen clearance, inflammation control, and improvement in organ involvement. At the same time, Day 28 and subsequent culture conversion rates and sustained conversion will still be retained as key secondary endpoints to evaluate the effect of different regimens on early bactericidal speed and long-term relapse/treatment failure.

Disseminated Mycobacterium avium complex (MAC) infection is a common opportunistic infection in patients with advanced HIV. It has insidious onset, a high organism burden, and frequent bacteremia. Early pathogen clearance speed is closely related to prognosis. Current treatment usually adopts a triple regimen consisting of a macrolide + ethambutol + a third agent (such as rifabutin). However, in patients with high organism burden, profound immunosuppression, or bacteremia, early culture conversion remains limited, and management of drug interactions and adverse events is complex. Fluoroquinolones

have in vitro activity against some mycobacteria, and short-term addition during the intensified phase may improve early bactericidal effect and culture conversion rate, but the true benefit and safety need to be verified by a randomized controlled trial.

3. Study Objectives and Hypotheses

3.1 Primary Objective

To evaluate whether the four-drug regimen, in which a fluoroquinolone (levofloxacin or moxifloxacin) is added to the standard triple therapy, can improve the Day 28 clinical symptom relief rate, and whether it provides clinically meaningful early symptom improvement compared with standard triple therapy.

3.2 Secondary Objectives

1. To compare early and intermediate microbiological responses between the two groups (Day 28 / Day 56 / Day 84 culture conversion rates, sustained conversion time);
2. To compare clinical outcomes between the two groups with respect to temperature, weight, symptom scores, imaging/organ involvement improvement, and length of hospitalization;
3. To compare all-cause mortality / MAC-related mortality, relapse rate, and treatment failure rate between the two groups;
4. To compare safety and tolerability between the two groups (grade ≥3 adverse events, serious adverse events, treatment discontinuation/dose reduction rates, QTc changes, etc.);
5. To explore factors influencing symptom relief and microbiological outcomes (baseline CD4, bacteremia and/or tissue NGS-positive site, comorbidities, concomitant IRIS, timing of ART initiation, etc.).

3.3 Study Hypothesis

The four-drug group will have a higher Day 28 symptom relief rate than the triple-drug group, without unacceptable severe toxicity or treatment interruption. A certain advantage is also expected in microbiological outcomes (culture/NGS negativity).

4. Study Design

(1) **Study type:** Prospective, multicenter, randomized, controlled, parallel-group clinical trial.

(2) **Allocation ratio:** 1:1.
(3) **Masking:** Open-label.
(4) **Study centers:** Multicenter; each center will enroll participants and conduct follow-up according to unified SOPs.
(5) **Sample size:** A total of 124 participants (62 per group).
(6) **Study duration:** Enrollment period of 12 months; each participant will be followed for at least 24 weeks, and follow-up will continue after Week 24 until discontinuation of study drugs.

5. Study Population

5.1 Target Population

People living with HIV who are diagnosed with disseminated MAC infection and plan to receive anti-MAC treatment.

5.2 Definition of Disseminated MAC

Confirmed DMAC: meeting any one of the following criteria:
(1) **Positive culture from a sterile site:** MAC isolated by culture from blood, bone marrow, or another sterile site (such as cerebrospinal fluid, pleural/peritoneal fluid, synovial fluid, pericardial/peritoneal specimens, etc.);
(2) **Molecular or sequencing evidence from tissue/sterile body fluid:** tissue or sterile body fluid specimens from a sterile site (such as liver, spleen, lymph node, intestinal wall, bone marrow, cerebrospinal fluid, pleural/peritoneal fluid, etc.) are positive for MAC by nucleic acid amplification testing or sequencing (including targeted sequencing and metagenomic NGS), and the quantity of result data/relative abundance is determined by the microbiology laboratory to represent clinically significant infection rather than contamination/background;
and accompanied by systemic disseminated manifestations (fever, weight loss, anemia, hepatosplenic/lymph node involvement, etc.), with the clinical team determining that treatment for disseminated MAC is indicated.

6. Inclusion and Exclusion Criteria

6.1 Inclusion Criteria

(1) Age ≥18 years;
(2) Confirmed HIV infection;
(3) Meets the diagnostic criteria for disseminated MAC (DMAC). At baseline, the source of

microbiological evidence that can be used for follow-up reassessment should be clarified as much as possible, including but not limited to: positive blood culture or positive culture from another sterile site/defined lesion specimen; and/or positive molecular evidence from a sterile site, such as nucleic acid testing/targeted sequencing/metagenomic NGS, considered by the microbiology laboratory to indicate clinically significant infection (not contamination/background). If a culture-positive source has not yet been obtained at baseline, enrollment is allowed, but a follow-up reassessment plan shall be established as soon as possible after randomization according to the principles of "same-source priority" and "blood culture priority," and the baseline evidence type, specimen source, and subsequent supplemental sampling/testing shall be recorded in the CRF;
(4) Planned to receive the protocol-specified standard triple therapy and has signed informed consent;
(5) Willing and able to undergo follow-up and specimen collection.

6.2 Exclusion Criteria

(1) Has already received an effective anti-MAC regimen for >14 days;
(2) History of severe allergy to a macrolide, ethambutol, rifabutin/rifampin, levofloxacin, or moxifloxacin;
(3) Previous/current evidence suggesting macrolide-resistant MAC (if susceptibility or molecular evidence is already available);
(4) Marked QTc prolongation (e.g., QTc >500 ms) or prior torsades de pointes;
(5) Uncorrected hypokalemia or hypomagnesemia (e.g., K <3.5 mmol/L or Mg below the lower limit of normal), or, in the investigator's judgment, a significant risk of arrhythmia;
(6) Prior severe fluoroquinolone-related adverse reactions (such as tendon rupture, severe peripheral neuropathy, severe central nervous system reactions, etc.);
(7) Known congenital long QT syndrome, or current use of Class I/III antiarrhythmic drugs that cannot be discontinued or replaced;
(8) Severe hepatic impairment (e.g., Child-Pugh C or ALT/AST >10×ULN and not controllable), severe renal insufficiency that cannot be managed with protocol-specified dose adjustment;
(9) Concomitant need for long-term use of drugs that significantly prolong the QT interval and cannot be replaced;
(10) Pregnancy or breastfeeding (if inclusion is considered, separate risk management and contraception requirements must be specified);
(11) Concomitant infection requiring non-protocol antimycobacterial therapy that would interfere with study evaluation (e.g., active tuberculosis requiring standard anti-TB treatment);
(12) Considered unsuitable for enrollment by the investigator (e.g., very poor adherence, expected survival <3 months, etc.).

7. Randomization and Stratification

(1) **Randomization method:** Central randomization system (IWRS/web-based randomization) or sealed opaque envelopes (with strict SOP required).
(2) **Stratification factors (recommended):**
a) Study center;
b) Baseline CD4 count (e.g., <50 vs ≥50 cells/μL);
c) Presence of bacteremia (blood culture positive vs no).

8. Interventions

8.1 Treatment Principles

In the four-drug group, a fluoroquinolone (levofloxacin or moxifloxacin) will be added to the standard triple regimen during the intensified phase (fixed as the first 8 weeks). The investigator must determine the specific fluoroquinolone at the time of randomization. In principle, it should not be changed during the first 8 weeks unless an adverse reaction or a clear contraindication occurs.

8.2 Treatment Arms

Group A: Standard Triple Therapy Group (Control)
(1) Macrolide (choose one): azithromycin or clarithromycin
(2) Ethambutol
(3) Third drug: rifabutin (or an equivalent third drug according to center standard; rifabutin is recommended as the preferred option to balance MAC treatment and drug interaction management)

Group B: Four-Drug Therapy Group (Experimental)
On the basis of the Group A triple regimen, add a fluoroquinolone (levofloxacin or moxifloxacin) (fixed during the first 8 weeks of the intensified phase; fixed within each individual to reduce bias).

Principles for fluoroquinolone selection (to be determined and recorded by the investigator): if there is a high risk of QT prolongation (borderline baseline QTc, prior arrhythmia, concomitant use of multiple QT-prolonging drugs, clear tendency toward electrolyte disturbances, etc.), levofloxacin may be preferred; if renal function is impaired or fluctuating (e.g., CrCl <50 mL/min) and QT risk is controllable, moxifloxacin may be selected. The selected agent should be maintained unchanged during the intensified phase whenever possible; if change is required due to adverse reactions/contraindications, the reason and timing of change shall be recorded, and sensitivity analysis will be performed according to the statistical plan.

Recommended doses:
(1) Azithromycin: 500–600 mg qd; or clarithromycin: 500 mg bid
(2) Ethambutol: 15 mg/kg qd
(3) Rifabutin: 300 mg qd (to be adjusted according to ART interactions)
(4) Fluoroquinolone (choose one): levofloxacin 500–750 mg qd (dose-adjusted according to renal function) or moxifloxacin 400 mg qd (generally no renal dose adjustment required; use with caution in patients with marked hepatic dysfunction)

8.3 ART Strategy

(1) In both groups: continue prior ART; or initiate ART within 2 weeks after starting anti-NTM treatment (preferably an integrase inhibitor-based regimen, with drug interaction assessment).
(2) Record the ART initiation date, regimen components, viral load, and CD4 dynamics; suspected IRIS shall be managed according to the predefined plan and recorded.

8.4 Concomitant Medication Management

(1) **Permitted:** prophylaxis for opportunistic infections such as PCP/toxoplasmosis, and symptomatic/supportive treatment.
(2) **Prohibited/use with caution:** drugs with strong interactions with study drugs that cannot be replaced; drugs with significant QT-prolonging effects require evaluation and monitoring.
(3) **Fluoroquinolone precautions:** levofloxacin/moxifloxacin should be administered separately from iron/calcium/magnesium/aluminum-containing products, antacids, etc. (recommended interval ≥4 hours) to avoid chelation-related reduction in absorption; if combined with other QT-prolonging drugs, intensified ECG monitoring is required.

9. Study Endpoints

9.1 Primary Endpoint

Day 28 (±3 days) clinical symptom relief rate.

Definition of clinical symptom relief: all of the following conditions must be met simultaneously:

1. **Defervescence:** axillary or oral temperature ≤37.3°C during the previous 48 hours, and no antipyretic/analgesic drugs used within 24 hours;
2. **Improvement in systemic symptoms:** using a prespecified symptom score scale (including fever-related symptoms, fatigue, night sweats, abdominal pain/diarrhea,

decreased appetite, etc., each scored 0–3), the total score at Day 28 has decreased by ≥50% from baseline, or all chief complaint symptom scores are ≤1;

3. **Improvement in general condition and nutritional status:** body weight has increased compared with baseline or at least is no longer decreasing (minor fluctuation is allowed, but "no progressive decline" is the minimum threshold), and the treating physician's overall assessment indicates clear improvement in general condition and better daily functioning;
4. **No escalation of anti-MAC treatment required:** no additional or replacement non-protocol core anti-MAC drug has been introduced before Day 28 due to "suspected or confirmed MAC progression";
5. **No death before Day 28.**

Failure to meet any one of the above criteria, or receipt of rescue treatment for suspected MAC progression, or death before D28 (±3 days), will all be classified as **not relieved**.

Note: The symptom score scale will be used as a separate appendix together with the CRF, and all centers will receive unified training before study initiation.

9.2 Secondary Endpoints

1. **Key secondary endpoint: microbiological outcomes:**
   (1) Day 28 (±3 days) culture/NGS negativity rate (using the same-source specimen as baseline or a prespecified alternative specimen);
   (2) Day 56 and Day 84 microbiological negativity rates;
   (3) Sustained culture conversion rate and time to conversion as defined in Section 17.2;
   (4) If feasible, dynamic changes in MAC sequence read counts or relative abundance in tissue or plasma mNGS may be recorded (exploratory endpoint).
   (5) Day 56/Day 84 culture negativity rates;
   (6) Time to culture conversion (from randomization date to the first date of sustained negativity).
2. **Clinical response:** time to defervescence, body weight change, symptom scores (fatigue/abdominal pain/diarrhea/night sweats, etc.), improvement in hemoglobin/inflammatory markers;
3. **Improvement in imaging/organ involvement** (evaluated using predefined assessable indicators according to type of organ involvement);
4. **All-cause mortality and MAC-related mortality at 24 weeks/48 weeks;**
5. **Relapse rate/treatment failure rate** (definitions required, such as persistently positive culture, clinical deterioration requiring regimen change, etc.);
6. **Safety:**
   (1) Incidence of adverse events (AEs) and serious adverse events (SAEs) (graded by CTCAE);
   (2) Grade ≥3 AEs and AEs leading to treatment discontinuation/dose reduction;
   (3) QTc prolongation and new-onset arrhythmia;

(4) Hepatic injury, renal injury, bone marrow suppression, optic neurotoxicity (ethambutol), tendinitis/central nervous system adverse reactions (fluoroquinolones), etc.

7. **Resistance/drug susceptibility changes** (exploratory endpoint, if feasible).

10. Visit and Assessment Schedule

10.1 Visit Time Points

· Screening period: D-7 to D0
· Baseline/randomization: D0
· Follow-up: D7 (±2), D14 (±3), D28 (±3), D56 (±7), D84 (±7), W24 (±14 days); W48 (±14 days); W60 (±14 days); W72 (±14 days).

10.2 Core Assessments at Each Visit (can be directly converted into CRF items)

· Symptoms/signs, vital signs, body weight
· Medication adherence and concomitant medication check
· Laboratory tests: complete blood count, liver and renal function, electrolytes (including K/Mg), ESR, CRP/procalcitonin (optional)
· ECG (recommended: baseline, D3-5, D7, D14, D28, and whenever palpitations/syncope occur)
· Visual acuity/color vision screening (ethambutol: baseline and every 4–8 weeks)
· Microbiology: baseline culture and D28 culture (primary endpoint), with repeat testing at D56/D84 according to protocol
· HIV-related: viral load, CD4 (baseline, W12, W24, etc.)
· Adverse event recording (CTCAE grade, causality assessment, management, and outcome)
· Collection of blood, urine, and stool samples

11. Safety Management and Rules for Discontinuation/Adjustment

11.1 Adverse Event Management

(1) If a grade ≥3 drug-related AE occurs: reduce dose/suspend/change treatment according to the predefined plan, and record the reason.

(2) If marked QTc prolongation or increased risk of ventricular arrhythmia occurs: preferentially discontinue the fluoroquinolone in use (if moxifloxacin is being used, moxifloxacin should be stopped first), and correct reversible factors (electrolyte disturbances, concomitant medications, etc.); cardiology consultation as needed.
(3) **Hepatotoxicity:** manage according to ALT/AST severity stratification; if necessary, suspend the rifamycin/macrolide and modify the regimen.
(4) **Suspected ethambutol-related optic neuritis:** stop the drug immediately and obtain ophthalmology consultation.
(5) **Fluoroquinolone-related tendon pain/tendinitis:** discontinue the drug and restrict physical activity.

11.2 SAE Reporting

· SAEs shall be reported to the study team and the ethics/pharmacovigilance-required institution(s) within 24 hours after awareness, in accordance with center requirements.

11.3 DSMB (Recommended)

An independent Data and Safety Monitoring Board (DSMB) will be established to periodically review SAEs and treatment discontinuations and, if necessary, recommend pausing enrollment or amending the protocol.

12. Sample Size Estimation

The total sample size has been set at 124 participants (62 per group).
· It is assumed that the Day 28 symptom relief rate in the control triple-therapy group is approximately P1, and that the four-drug regimen will increase this to P2 (for example, by 15–20 percentage points);
· Using a two-sided $\alpha=0.05$ and 80% power, and allowing for approximately 10% loss to follow-up/withdrawal;
· It is estimated that approximately 62 participants per group, 124 in total, will meet the comparison requirements for the primary endpoint.

13. Statistical Analysis Plan

13.1 Definition of Analysis Sets

1. **ITT Set (Intention-to-Treat Set)**
   · Includes all participants who complete randomization and are assigned to either treatment group;

· Will serve as a supplementary sensitivity analysis population for some secondary endpoints and safety analyses.

2. **mITT Set (Modified Intention-to-Treat Set; recommended as the primary analysis set for the primary endpoint)**
   · Inclusion criteria:
   a) Meets the diagnostic criteria for "confirmed DMAC" at baseline (MAC confirmed positive by sterile-site culture and/or NGS, with systemic disseminated manifestations);
   b) Has received at least 1 dose of protocol-specified anti-MAC treatment;
   c) Has evaluable information on clinical symptom relief at D28 (±3 days), or can be classified according to prespecified missing data handling rules;
   · The mITT set will be used for the primary analysis of the **primary endpoint (Day 28 clinical symptom relief rate)** and for the main analyses of key secondary endpoints.
3. **PP Set (Per-Protocol Set)**
   · Based on the mITT set, additionally meeting:
   a) No major protocol deviation (such as serious violation of inclusion/exclusion criteria, major medication deviation, severe missingness of key visits, etc.; detailed criteria to be predefined in the statistical analysis plan);
   b) Completion of the D28 key visit and core follow-up during the main treatment period;
   · The PP set will be used for sensitivity analyses of the primary endpoint and key secondary endpoints to assess robustness of results.
4. **Safety Set**
   · All participants who receive at least 1 dose of study drug (any anti-MAC study regimen drug);
   · Used for safety analyses (AE/SAE, adverse drug reactions, laboratory abnormalities, QTc changes, etc.).

13.2 Primary Endpoint Analysis

1. **Primary analysis population**
   · The mITT set will be used as the primary analysis population;
   · Sensitivity analyses will also be performed in the ITT set and PP set.
2. **Between-group comparison method**
   · Using treatment group (control triple-drug group vs fluoroquinolone four-drug group) as the independent variable, compare the difference in **Day 28 (±3 days) clinical symptom relief rate** between the two groups;
   · The **chi-square test** will be used for between-group comparison; if expected cell counts are insufficient, the **Fisher exact test** will be used.
3. **Effect size estimation**
   · Calculate the **absolute difference in relief rates (Risk Difference, RD)** between the two groups and its 95% confidence interval;
   · Calculate the **relative risk (Risk Ratio, RR)** and its 95% confidence interval;

· If needed, the **odds ratio (OR)** and 95% confidence interval may also be provided (from a logistic regression model).

4. **Multivariable adjusted analysis**
   · A multivariable logistic regression model will be constructed, with achievement of clinical symptom relief at D28 (yes/no) as the dependent variable and treatment group as the main independent variable;
   · The following prespecified covariates will be included for adjustment:
   a) Study center (as a stratification factor or dummy variable);
   b) Baseline CD4 stratum (e.g., <50 vs 50–100 cells/μL);
   c) Baseline microbiological type (culture positive only vs NGS-dominant vs both culture and NGS positive; may be simplified depending on center conditions);
   d) Baseline total symptom score (continuous variable or stratified by median);
   e) Presence of bacteremia and other important prognostic factors;
   · The adjusted OR and 95% CI will be reported, together with comparison against unadjusted results.
5. **Sensitivity analyses**
   · **Analysis population sensitivity analysis:** repeat the primary endpoint analysis in the ITT set and PP set separately and compare consistency of results;
   · **Missing data sensitivity analysis:**
   a) The primary analysis will use a conservative strategy in which "missing = not relieved";
   b) In sensitivity analyses, multiple imputation or best-case/worst-case scenario analyses may be used to assess the impact of missingness patterns on the results.

13.3 Secondary Endpoint Analysis

Secondary endpoints include microbiological outcomes, time-to-event outcomes, changes in continuous variables, and safety outcomes. No uniform multiplicity adjustment will be applied (or this may be specified separately in the SAP). The following are the recommended main analysis methods.

1. **Microbiological outcomes (culture/NGS negativity)**
   · **D28, D56, and D84 microbiological negativity rates (key secondary endpoints):**
   a) Analysis population: participants in the mITT set with evaluable baseline microbiological positivity (culture and/or NGS);
   b) Method: same as for the primary endpoint, using chi-square test/Fisher exact test to compare negativity rates between groups, reporting RD, RR, and 95% CI;
   c) A logistic regression model may be constructed to adjust for covariates such as center, CD4 stratum, and baseline organism burden.

· **Time to microbiological conversion:**
a) Defined as the time (in days) from initiation of study treatment to first microbiological negativity (culture and/or NGS);

b) Kaplan–Meier curves will be used to describe the distribution of time to conversion in the two groups, with comparison by the log-rank test;
c) A Cox proportional hazards model will be used to estimate the effect of treatment group on the event of "microbiological conversion" (hazard ratio [HR] and 95% CI), with adjustment for covariates such as CD4 and baseline bacteremia/NGS burden.

2. **Long-term prognosis and time-to-event outcomes**
   · **All-cause death, MAC-related death, MAC relapse, treatment failure**, and similar events:
   a) Kaplan–Meier methods will be used to estimate cumulative event risk;
   b) The log-rank test will be used to compare differences between groups;
   c) Cox regression models will be used to estimate HRs and 95% CIs, adjusting for prespecified covariates (such as age, CD4, baseline symptom severity, microbiological type, etc.).
3. **Continuous variables (body weight, laboratory parameters, changes in symptom scores, etc.)**
   · For continuous variables measured repeatedly at baseline and multiple follow-up time points (such as body weight, laboratory parameters, total symptom score, etc.):
   a) **Between-group comparison at the same time point:** if data are approximately normally distributed, use the **t test**; if the distribution is skewed or contains outliers, use the **Wilcoxon rank-sum test (Mann–Whitney U test)**;
   b) **Analysis of change from baseline (Δ) to each time point:** the same methods may be used;
   c) For variables repeatedly measured over time (such as total symptom score, body weight, laboratory parameters), a **linear mixed-effects model** or **generalized estimating equations (GEE)** is recommended, with participant as a random effect and time, treatment group, and time*treatment interaction as fixed effects, to assess differences in longitudinal trends between groups.
4. **Safety endpoints**
   · Safety analyses will be conducted in the **Safety Set**;
   · **Categorical variables** (AE/SAE incidence, incidence of specific adverse reactions, discontinuation rate, etc.): descriptive analysis (counts and percentages) will be used; between-group comparisons may use the chi-square test or Fisher exact test;
   · **Continuous variables** (such as liver/renal function indices, QTc interval): descriptive statistics will be used (mean ± standard deviation, median [IQR], etc.), with between-group comparison methods as above for continuous variables;
   · Key safety indicators (such as grade ≥3 drug-related AEs, serious arrhythmia, marked decline in renal function, etc.) may be presented in separate summary tables.

13.4 Missing Data

13.4.1 General Principles

Every effort will be made to minimize missing data, including: arranging reminders within each visit window, allowing make-up sampling/examinations within the window, recording reasons for incompletion (loss to follow-up, refusal, specimen contamination/non-qualification, transfer to another hospital/death, etc.), and fully documenting the reason and date of missingness in the CRF. Missing data handling will follow the principles of "prespecified, transparent methods, and robustness verified by sensitivity analyses."

13.4.2 Prespecified Handling of Missing Primary Endpoint Data (Primary Analysis)

The primary endpoint is **D28 (±3 days) culture conversion to negative**. Handling of missing primary endpoint data (including no sampling, culture contamination/uninterpretable result, result not returned, etc.) is as follows:

1. **Conservative approach (default for primary analysis):** if the D28 (±3 days) culture result is missing, it will be classified as **not converted to negative** in the primary endpoint analysis (failure imputation).
2. **Death/early discontinuation for disease progression/rescue treatment:** if death occurs before D28 (±3 days), or rescue treatment/non-protocol intensification is given because of "insufficient efficacy/disease progression," making an interpretable D28 culture result unavailable, the primary endpoint will likewise be classified as **not converted to negative**.
3. **Missing only for non-efficacy reasons** (for example, failure to return to hospital due to transportation issues, difficulty with specimen collection, etc.): this will still be handled according to the conservative rule in the primary analysis, but its impact will be explored in sensitivity analyses (see 13.4.4).

Note: The above conservative rule helps avoid overestimation of the culture conversion rate due to missing data and is consistent with the commonly used "conservative tendency" principle in clinical trials.

13.4.3 Handling of Missing Secondary Endpoint Data (Overview)

1. **Continuous variables** (such as laboratory parameters): mixed-effects models/repeated measures models will be preferred (without explicit imputation under the MAR assumption), or multiple imputation will be performed when necessary.
2. **Time-to-event outcomes** (such as "time to conversion"): if conversion is not observed, data will be handled as **censored**, with censoring time defined as the date of the last evaluable culture.

13.4.4 Sensitivity Analyses (to Verify Robustness of the Primary Outcome)

To assess the impact of missing data handling on conclusions, the following sensitivity analyses are prespecified (at least 2 of these should be conducted for stronger ethical/statistical rigor):

1. **Complete-case / Evaluable analysis**
   Include only participants with an interpretable culture result at D28 (±3 days), and recalculate and compare culture conversion rates between the two groups.
2. **Multiple imputation (MI)**
   Under the assumption that the missing data mechanism is MAR, MI will be performed for the D28 culture conversion outcome (e.g., logistic regression imputation). The imputation model is recommended to include:
   · Grouping factor (standard triple therapy vs four-drug fluoroquinolone intensified therapy)
   · Baseline culture source (blood/bone marrow/other), baseline organism burden (if available), baseline CD4, HIV viral load (if available)
   · Baseline anemia/inflammatory markers (Hb, CRP, etc.), concomitant opportunistic infection/tuberculosis treatment, timing of ART initiation
   · Key adherence/early discontinuation status
   Generate ≥20 imputed datasets, pool estimates, and report the pooled effect size and 95% CI.
3. **Best–worst / Worst–best scenario analyses**
   · Scenario 1 (favoring the four-drug group): missing data in the four-drug group are counted as "converted to negative," and missing data in the triple-drug group are counted as "not converted to negative";
   · Scenario 2 (favoring the triple-drug group): missing data in the four-drug group are counted as "not converted to negative," and missing data in the triple-drug group are counted as "converted to negative";
   Assess whether the conclusions change.
4. **Supplementary analysis: stratification by reason for missingness**
   Missing data will be classified as "efficacy-related" (death/rescue treatment/discontinuation for insufficient efficacy) and "non-efficacy-related" (loss to follow-up/administrative reasons/contamination), and the above methods will be applied separately for assessment, with differences reported.

13.4.5 Assessment and Reporting of Missing Data Mechanisms

The proportion of missing data at each visit, the distribution of reasons for missingness, and the association between baseline characteristics and missingness will be summarized

(to evaluate the likelihood of MCAR/MAR/MNAR). All missing data handling methods and results will be reported alongside the primary analysis.

14. Data Management and Quality Control

(1) eCRF and paper CRF double entry will be used; logic checks and audit rules will be established;
(2) Unified SOPs: specimen collection, transport, culture, and identification procedures (central laboratory quality control is recommended);
(3) Monitoring plan: site initiation visits, regular remote/on-site monitoring, and closeout audit;
(4) Key bias control measures: random allocation, stratification, blinded endpoint assessment, and inter-center training and consistency assessment.

15. Ethical Considerations

(1) The study will comply with the Declaration of Helsinki and relevant GCP requirements;
(2) Participants will sign informed consent; potential benefits and risks will be emphasized (especially hepatotoxicity, QTc prolongation, optic neurotoxicity, drug interactions, tendinitis, etc.);
(3) Protection of personal privacy and data de-identification;
(4) In case of harm, management will follow the center's clinical trial insurance/treatment process (if applicable).

16. Trial Organizational Structure

(1) **Lead institution:** Shanghai Public Health Clinical Center
(2) **Investigator meetings and training:** initiation meeting, monthly progress reports, and semiannual study meetings
(3) **DSMB:** expert panel composed of participating study centers
(4) **Laboratory:** each study center's laboratory results will be accepted from the respective centers
(5) **Statistical unit:** independent statistician/statistical center

17. Key Definitions

17.1 Diagnostic Criteria for Disseminated MAC (DMAC)

1. **Confirmed DMAC:** meeting any one of the following criteria:
   (1) **Positive culture from a sterile site:** MAC isolated by culture from blood, bone

marrow, or another sterile site (such as cerebrospinal fluid, pleural/peritoneal fluid, synovial fluid, pericardial/peritoneal specimens, etc.);
(2) **Molecular or sequencing evidence from tissue/sterile body fluid:** tissue or sterile body fluid specimens from a sterile site (such as liver, spleen, lymph node, intestinal wall, bone marrow, cerebrospinal fluid, pleural/peritoneal fluid, etc.) are positive for MAC by nucleic acid amplification testing or sequencing (including targeted sequencing and metagenomic NGS), and the quantity of result data/relative abundance is determined by the microbiology laboratory to represent clinically significant infection (not contamination/background);
and accompanied by systemic disseminated manifestations (fever, weight loss, anemia, hepatosplenic/lymph node involvement, etc.), with the clinical team determining that treatment for disseminated MAC is indicated.

2. **Probable/highly suspected DMAC (Probable DMAC; may be recorded during screening, with enrollment subject to protocol requirements):**
(1) Typical systemic disseminated manifestations plus imaging/laboratory findings suggestive of DMAC (such as elevated ALP, extensive hepatosplenic/lymph node involvement, etc.);
(2) and MAC isolated or molecularly detected from a non-sterile site (such as sputum, stool, bronchoalveolar lavage fluid);
(3) but without yet obtaining positive culture from a sterile site/blood/bone marrow or tissue evidence; before enrollment, efforts should be made to complete a "microbiological evidence source that can be used for follow-up reassessment" (culture or sterile-site molecular/sequencing evidence), and this shall be recorded in the CRF.
(4) For cases in which NGS is the primary evidence, if the initial specimen is from a sterile site and subsequent same-source specimens or blood cultures can be repeatedly collected, such cases will be included in the mITT primary analysis; if only a single positive NGS result is available and repeat sampling cannot be performed, such cases will still be recorded in the ITT set and evaluated separately in sensitivity analyses.
3. **Definition of baseline "evaluable culture positivity"** (for culture-related secondary endpoints/subgroup analyses): refers to positive blood culture or positive culture from a defined lesion/sterile-site specimen obtained before randomization (or on D0), preferably blood culture, and the same specimen source can be repeatedly sampled during follow-up. This is used to ensure evaluability of microbiological outcomes such as culture negativity rate and sustained conversion.

17.2 Definition of Culture Conversion

The primary endpoint of this study is **D28 clinical symptom relief**; the definitions of culture conversion, treatment failure, and relapse in Sections 17.2–17.4 are used for microbiology-related secondary endpoints and exploratory analyses.

1. **Primary microbiological endpoint: D28 culture conversion (single conversion to negative)**
   · **Definition:** the culture result of the repeat specimen at Day 28 (±3 days) is negative; the specimen should preferentially be from the same source as baseline (e.g., if baseline blood culture was positive, repeat blood culture is preferred; or if baseline positivity was from a defined lesion/sterile site, a specimen from the same site/type should be retested).
   · **Sampling window:** D25–D31.
   · **Specimen priority** (recommended for SOP): blood culture > baseline-positive sterile-site/defined lesion specimen > other alternative specimen. If alternative specimens are allowed, this must be prespecified in the protocol and sensitivity analysis must be performed in the statistical analysis.
2. **Sustained culture conversion** (for secondary endpoints/time to conversion)
   · **Definition:** during treatment, at least 2 consecutive negative cultures are obtained (recommended interval ≥7 days) from the same source (or according to prespecified alternative rules); the date on which this criterion is first met is defined as the "conversion date."
   · **Time to conversion:** from the date of randomization (D0) to the date first meeting the definition of sustained conversion.
3. **Rules for interpretation of culture results**
   · **Contaminated/uninterpretable:** if the culture report indicates contamination or inability to interpret, it will in principle be treated as missing, and repeat sampling should be performed as soon as possible; missing primary endpoint data will be handled according to the prespecified missing data rules in the statistical analysis plan.
   · **Reversion after conversion:** if the same-source culture becomes positive again after sustained conversion has been achieved, this will be recorded as "reversion" and entered into the adjudication process for relapse/treatment failure (see 17.3/17.4).

17.3 Criteria for Treatment Failure

Meeting any one of the following criteria will be classified as **treatment failure** (the specific reason may be checked in the CRF):

1. **Microbiological failure**
   After sufficient drug exposure time has been achieved following treatment initiation (with D56 as the key time point, consistent with the follow-up design), any of the following occurs: persistently positive cultures (for example, still positive at D56 and thereafter), or failure to achieve the definition of sustained conversion together with clinical/laboratory evidence that the disease has not improved.
2. **Clinical failure** (investigator-determined, with objective evidence required)
   · Persistent or worsening symptoms/signs (such as persistent fever, weight loss, worsening anemia/inflammatory markers, etc.), considered by the investigator to be

related to active MAC; or
· Progression of imaging/organ involvement (worsening under the same assessment method); or
· Need to add or switch to a non-protocol core anti-MAC drug (for example, adding an injectable aminoglycoside or changing to another second-line regimen) due to insufficient efficacy.

3. **Death due to MAC**
   · The investigator and endpoint adjudication body (e.g., DSMB/expert panel) jointly determine that the primary cause of death is related to active MAC.

Note: If discontinuation/change of treatment is primarily due to adverse reactions and there is no clear evidence of insufficient efficacy, it will not be directly classified as treatment failure, but must be recorded under "protocol deviation/safety" and handled in the PP/sensitivity analyses.

17.4 Criteria for Relapse (Relapse/Recurrence)

After "sustained conversion" has been achieved, meeting any one of the following will be classified as **relapse** (it is recommended that at least one criterion be met, and obvious laboratory contamination be excluded):

1. **Microbiological relapse:** the same-source culture (or culture according to prespecified alternative rules) becomes positive again, consistent with recurrence of clinical symptoms/inflammatory markers/imaging findings;
2. **Clinical relapse:** although a positive culture cannot be obtained, clinical recurrence highly consistent with MAC occurs, and the investigator restarts or intensifies anti-MAC treatment for this reason;
3. If feasible, **strain homology/drug susceptibility pattern consistency** may be added as exploratory evidence supporting relapse (not required).

Relapse assessment window: systematically assessed at W24 and, if applicable, W48 follow-up.

17.5 Criteria for IRIS (Immune Reconstitution Inflammatory Syndrome) (MAC-IRIS)

In line with the protocol requirement that "suspected IRIS after ART initiation shall be monitored and recorded," the following operational definition is recommended:

1. **Time window:** usually occurs within 3 months after ART initiation (0–12 weeks may be used as the primary observation window; if occurring after 12 weeks, it may still be recorded as "late-onset IRIS [possible]").

2. **Classification**
   · **Unmasking MAC-IRIS:** after ART initiation, new MAC-related inflammatory manifestations appear that were previously unrecognized, with MAC confirmed or highly suspected.
   · **Paradoxical MAC-IRIS:** MAC has already been diagnosed and is under treatment, but short-term clinical deterioration/increased inflammation occurs after ART initiation, not caused by treatment failure or a new pathogen.
3. **Core adjudication elements** (recommended to require A+B and fulfillment of C)
   A. New or worsened inflammatory clinical manifestations after ART initiation: rebound fever, enlargement of lymph nodes/intra-abdominal lesions, worsening hepatosplenic involvement, inflammatory bone marrow manifestations, etc.;
   B. Immunologic/virologic evidence supporting immune reconstitution: rise in CD4 and/or decline in HIV viral load (based on testing available at the center);
   C. Exclusion of other causes:
   · Exclude "true treatment failure" caused by insufficient anti-MAC drug exposure/adherence problems;
   · Exclude new opportunistic infection, drug fever, drug toxicity (such as liver injury), malignancy, etc.
4. **IRIS severity** (for recording and analysis)
   · **Mild/moderate:** controllable without hospitalization or with only short-term symptomatic treatment;
   · **Severe:** requiring hospitalization, systemic corticosteroids/invasive intervention, or associated with organ dysfunction.

17.6 AE/SAE Definitions and CTCAE Version

(1) **Definition of adverse event (AE)**
· Any unfavorable medical event occurring during or after use of study drugs, regardless of whether it has a causal relationship with the study drugs.

(2) **Definition of serious adverse event (SAE)** (meeting any one of the following)
· Results in death;
· Is life-threatening;
· Requires hospitalization or prolongation of hospitalization;
· Results in persistent or significant disability/incapacity;
· Congenital anomaly/birth defect;
· Important medical event considered by the investigator to require intervention to prevent one of the above outcomes.

(3) **Grading standard**
· **NCI-CTCAE v5.0** will be used as the uniform grading standard.

(4) **Adverse events of special interest (AESI)** (recommended for inclusion to facilitate uniform monitoring)

· **QTc prolongation/arrhythmia:** for example, QTc >500 ms or increase >60 ms from baseline, or occurrence of torsades de pointes/ventricular arrhythmia (consistent with the protocol's QT risk monitoring logic);
· Hepatotoxicity/nephrotoxicity, bone marrow suppression;
· Ethambutol-related optic neurotoxicity;
· Fluoroquinolone-related tendinitis/tendon rupture, peripheral neuropathy, central nervous system reactions, dysglycemia, etc.