BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-094366
Article Type:	Protocol
Date Submitted by the Author:	29-Sep-2024
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Keywords:	CHEMOTHERAPY, Clinical Relevance, Clinical Trial, ONCOLOGY, Respiratory tract tumours < ONCOLOGY

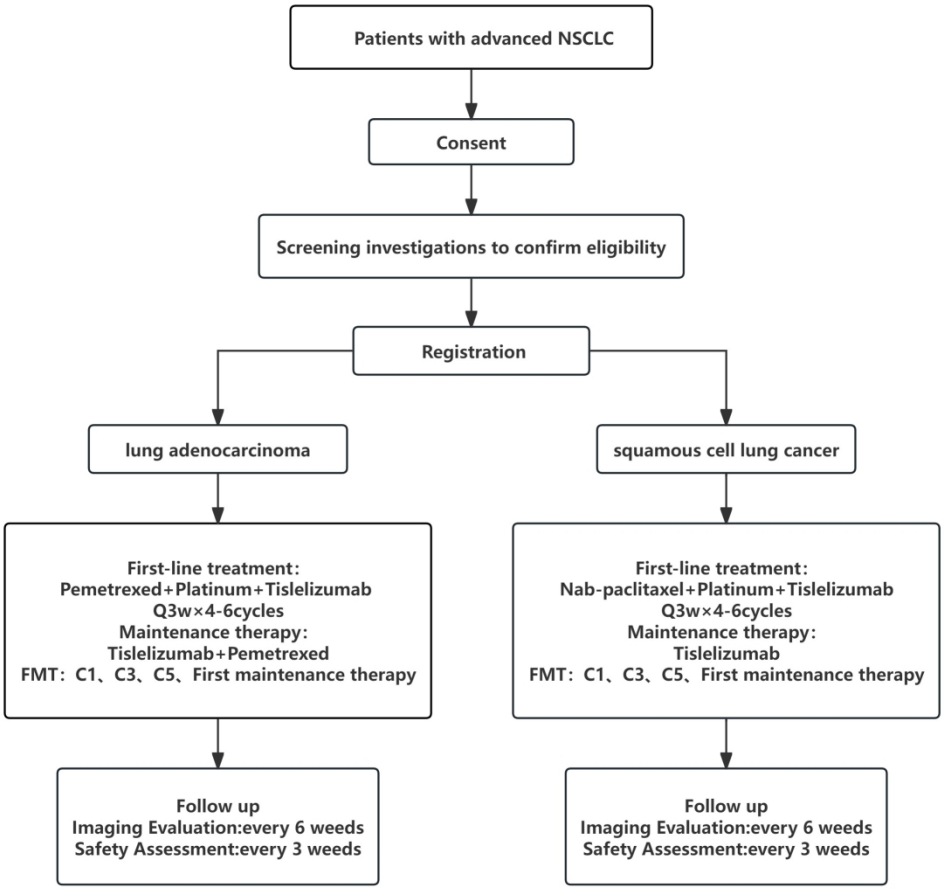

Figure 1 The participant pathway of the clinical trial

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FMT combined with first-line treatment for driver-gene negative advanced NSCLC: study protocal for a prospective, multicenter, single-arm exploratory trial

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ABSTRACT

Introduction: Chemotherapy combined with immunotherapy is the standard first-line treatment for driver-gene negative advanced non-small cell lung cancer (NSCLC). However, due to the immune microenvironment imbalance and immune status impairment caused by repeated chemotherapy, as well as the primary or secondary resistance to immune checkpoint inhibitors, the efficacy of immunotherapy combined with chemotherapy remains unsatisfactory. Recent studies have shown that fecal microbiota transplantation (FMT) can modulate the intestinal microflora, affecting the tumor immune microenvironment, and further enhancing the efficacy of tumor immunotherapy.

Methods and analysis: FMT-JSNO-02 (NCT06403111) is a prospective, multicenter, single-arm exploratory study. It is planned to include 62 cases of previously untreated driver-gene negative, ECOG PS 0-1, PD-L1<50% advanced NSCLC patients, who will

be given FMT by orally ingested stool capsules on the basis of first-line treatment of chemotherapy combined with immunotherapy. The safety and efficacy of this treatment regimen will be evaluated, with the primary endpoint being the 12-months progression-free survival (PFS) rate.

Ethics and dissemination: Approved by the Ethics Committee of Changzhou No.2 People's Hospital (number [2024] YLJSA005).

Trial registration number: NCT06403111.

Strengths and limitations of this study:

- This is a multicenter prospective study.
- Our study aims to regulate intestinal flora and introduce FMT into the first-line treatment of lung cancer for the first time.
- The sample size in this study is relatively small.
- This is a single-arm study rather than a randomized controlled study.

keywords: Fecal microbiota transplantation, Non Small Cell Lung Cancer, Chemotherapy, Immunotherapy

A word count: 3205

1. Introduction

Current Situation and Dilemmas of Immunotherapy for NSCLC

Lung cancer is the most common malignancy worldwide and remains the leading cause of cancer-related mortality, accounting for 18.7% of all cancer deaths.[1]As the main pathological subtype of lung cancer, the majority of NSCLC patients present with local spread or distant metastasis upon diagnosis, losing the opportunity for surgery and having a very poor prognosis. In recent years, immune checkpoint inhibitors (ICIs), particularly those targeting the programmed death receptor 1 (PD-1) and its ligand (PD-L1), markedly improved the treatment outcomes and becoming an effective treatment strategy for NSCLC.

At present, the combination of chemotherapy and immunotherapy has become the standard first-line treatment for patients with advanced NSCLC due to extensive clinical studies conducted on Pembrolizumab and Nivolumab. Tislelizumab developed in China has also shown remarkable efficacy. In patients with lung adenocarcinoma, the addition of Tislelizumab to chemotherapy resulted in significantly prolonged median progression-free survival (mPFS) compared to chemotherapy alone (9.7 versus 7.6 month), with a 12-month PFS rate of 31.3%.[2] Similarly, in patients with squamous cell lung cancer, the mPFS for Tislelizumab plus chemotherapy group was also higher than that of chemotherapy alone (7.6 months versus 5.5 months).[3]

Although ICIs significantly improved the survival of patients with advanced NSCLC, primary or acquired immune resistance inevitably occurs in clinical practice. Existing research suggests that primary immune resistance is related to the dysfunction in tumor antigen processing and presentation, insufficient T cell infiltration in the tumor microenvironment (TME), and over expression of suppressive immune cells.[4] Acquired immune resistances is related to adaptive changes that occur in tumor cells and the TME

during exposure to immunotherapy.[5]

Nowadays, a growing body of research suggests that gut flora may be able to reverse immune resistance. For instance, gut microbiota can regulate the infiltration of immune cells in the TME by modulating innate and adaptive immunity. They can also reprogram the TME through their reactive metabolites or secretions, affecting the efficacy of ICIs and even reversing immune resistance.[6] Bifidobacterium can promote the maturation of dendritic cells and increase the infiltration of $CD8^+$ T cells in the TME as well.[7]

Similarily, research on the improvement of tumor immunotherapy efficacy by gut microbiota has been widely verified. Zitvoge found that the antitumor effects of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade depended on distinct Bacteroides species. Tumors in antibiotic-treated or germ-free mice did not respond to CTLA-4 blockade. This defect was overcome by gavage with B. fragilis, by immunization with B. fragilis polysaccharides, or by adoptive transfer of B. fragilis-specific T cells.[8] At the same time, Sivan et al. found that mice orally administered Bifidobacterium could achieve the same anti-tumor effect as PD-L1 monoclonal antibodies, and the combination almost completely inhibited tumor growth.[7] Therefore, modulating gut microbes has the potential to further improve the treatment outcomes for patients receiving immunotherapy.

Current Status of Fecal Microbiota Transplantation (FMT)

The gut microbiota is susceptible to various factors, including the use of broad-spectrum antibiotics, repeated chemotherapy and other treatments. Common methods to regulate the intestinal microbiota include dietary intervention, probiotics, prebiotics, and FMT. Among these approaches, FMT stands as the most established technique.[9] FMT is the transplantation of functional flora from the stool of a healthy person into the intestine of a

patient in a certain way. This method mainly includes two ways: injecting microflora fluids and swallowing capsules. Among them, FMT by orally ingested stool capsules (capsulized FMT) has the characteristics of a wide range of application, high acceptance degree, relative economy and convenience.[10]

FMT has been shown to ugment the antitumor effect of ICIs and overcome resistance to immunotherapy. In a study focused on 15 melanoma patients resistant to anti-PD-1 therapy, rechallenge with FMT and Pembrolizumab showed an objective responses in 3 out of 15 patients, and these 3 patients kept stable disease (SD) for more than 12 months.[11] In a clinical trial (NCT04264975), researchers performed FMT with anti-PD-1 inhibitors in 13 patients with anti-PD-1 refractory advanced solid cancers. The results showed that FMT induced sustained microbiota changes and clinical benefits in 6 patients, achieving an objective response rate (ORR) of 7.7% and a disease control rate (DCR) of 46.2%.[12] As a result, FMT holds great potential to reverse immune resistance and enhance the efficacy of immunotherapy.

Hence, we conduct such a prospective, exploratory study in order to verify whether FMT combined with first-line treatment can prolong the treatment efficacy for driver-gene negative NSCLC. By observing the adverse events and measuring the 12-months PFS rate, the safety and efficacy of this therapy will be verified. It may be possible to provide a more effective treatment option for NSCLC patients.

2. Methods

2.1 Study Design

This is a prospective, multicenter, single-arm exploratory study aimed at evaluating the safety and efficacy of FMT combined with first-line treatment for driver-gene negative NSCLC patients. A total of 62 patients with driver-gene negative, ECOG PS 0-1, and

PD-L1<50% who have not received prior treatment are expected to be included in this study without grouping. Patients with squamous cell lung cancer will receive Nab-paclitaxel + Platinum + Tislelizumab for 4-6 cycles and then Tislelizumab maintenance therapy. Patients with lung adenocarcinoma will receive Pemetrexed + Platinum + Tislelizumab for 4-6 cycles and then Tislelizumab + Pemetrexed maintenance therapy. All the enrolled patients or their designated agents will sign the informed consent form within 3 days before starting treatment. The study was approved by the ethics committee of Changzhou No.2 People's Hospital (number [2024] YLJSA005), and was registered at ClinicalTrials.gov (NCT06403111). See Figures 1 for details

2.2 Patient Population

Inclusion criteria:

- The subjects voluntarily joined the study and were able to sign the informed consent with good compliance.
- Age 18-80 years old (when signing the informed consent form).
- Patients with histologically or cytologically proven locally advanced (ⅢB/ⅢC), metastatic, or recurrent (stage IV) NSCLC who are inoperable and unable to receive radical concurrent chemoratherapy, according to the International Association for the Study of Lung Cancer and the American Joint Committee on Cancer Classification, 8th Edition TNM Classification of Lung cancer.
- Have not received systemic intravenous anti-tumor therapy before, and the driver-gene is negative.
- PD-L1 expression <50%.
- According to the solid tumor efficacy evaluation criteria (RECIST 1.1), there is at least one radiographically measurable lesion. That is, in CT or MRI detection, the longest diameter of a single lesion was ≥10mm, or the pathological enlargement of a single lymph node was ≥15mm.
- The physical status score of Eastern Tumor Collaboration Group (ECOG) was 0-1.

- Expected survival >3 months.
- Have adequate organ and bone marrow function, laboratory tests within 7 days prior to enrollment meet the following requirements (no blood components, cell growth factors, albumin and other corrective drugs are allowed within 14 days prior to obtaining laboratory tests), as follows: 1) Blood routine: absolute neutrophil count (ANC) ≥1.5×109/L, platelet (PLT) ≥75×109/L, hemoglobin (HGB) ≥90 g/L (no blood transfusion or erythropoietin dependence within 14 days). 2) Liver function: serum total bilirubin (TBIL) ≤2 times the upper limit of normal (ULN). Alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) ≤ 5x ULN, serum albumin ≥28 g/L. alkaline phosphatase (ALP) ≤5×ULN. 3) Renal function: serum creatinine (Cr) ≤1.5×ULN, or creatinine clearance ≥50 mL/min (using the standard Cockcroft-Gault formula) : Urine routine results showed urinary protein < 2+. For patients with urine protein ≥2+ at baseline, 24-hour urine collection and 24-hour urine protein quantification < 1g should be performed. 4) Coagulation function: International standardized ratio (INR) or prothrombin time (PT) ≤1.5 times ULN. If the subject is receiving anticoagulant therapy, as long as the INR is within the intended range of anticoagulant drug use.
- For female subjects of reproductive age, a urine or serum pregnancy test should be performed and the result is negative 3 days prior to receiving the initial study drug administration.
- Subjects and their sexual partners are required to use a medically approved contraceptive method (such as an intrauterine device (IUD), contraceptive pill, or condom) during the study treatment period and for 6 months after the end of the study treatment period.

Exclusion criteria:

- Currently participating in an interventional clinical study or receiving another investigational drug or investigational device within 4 weeks prior to initial dosing.

- Received proprietary Chinese medicines with anti-tumor indications or immunomodulatory drugs (thymosin, interferon, interleukin, etc.) within 2 weeks before the first administration, or received major surgical treatment within 3 weeks before the first administration.
- Class III - IV congestive heart failure (New York Heart Association classification), poorly controlled and clinically significant arrhythmias.
- Any arterial thrombosis, embolism or ischemia, such as myocardial infarction, unstable angina pectoris, cerebrovascular accident or transient ischemic attack, occurred within 6 months before treatment.
- Known allergic reaction to the drug in this study.
- Patients requiring long-term systemic use of corticosteroids. Patients with chronic obstructive pulmonary disease (COPD) or asthma requiring intermittent use of bronchodilators, inhaled corticosteroids, or local corticosteroids could be enrolled.
- Symptomatic central nervous metastases. Patients with asymptomatic brain metastases (BMS) or BMS whose symptoms are stable after treatment are eligible to participate in this study if they meet all of the following criteria: measurable lesions outside the central nervous system. No midbrain, pontine, cerebellum, meninges, medulla oblongata or spinal cord metastasis. Maintain clinical stability for at least 2 weeks. Stop hormone therapy 3 days before the first dose of the study drug.
- There is an active infection requiring treatment or systemic anti-infective drugs have been used in the week prior to the first dosing.
- Has not fully recovered from toxicity and/or complications caused by any intervention before starting treatment (i.e., ≤ grade 1 or baseline, excluding weakness or hair loss).
- Known history of human immunodeficiency virus (HIV) infection (i.e. HIV 1/2 antibody positive).
- Untreated active hepatitis B (defined as HBsAg positive and HBV-DNA copy

number detected greater than the upper limit of normal value in the laboratory of the study center). Note: Hepatitis B subjects who meet the following criteria can also be enrolled: HBV viral load <1000 copies /ml (200 IU/ml) before initial administration, subjects should receive anti-HBV therapy throughout the study chemotherapy drug treatment to avoid viral reactivation. 2) For subjects with anti-HBC (+), HBsAg (-), anti-HBS (-) and HBV viral load (-), prophylactic anti-HBV therapy is not required, but close monitoring of viral reactivation is required.

- Active HCV-infected subjects (HCV antibody positive and HCV-RNA levels above the lower limit of detection).
- Received live vaccine within 30 days prior to the first dose (cycle 1, day 1). Note: Injectable inactivated virus vaccine against seasonal influenza is permitted for 30 days prior to initial administration. However, live attenuated influenza vaccines administered intranasally are not permitted.
- Pregnant or lactating women.
- Medical history or evidence of disease that may interfere with test results, prevent participants from fully participating in the study, abnormal treatment or laboratory test values, or other conditions that the investigator considers unsuitable for enrollment. The Investigator considers other potential risks unsuitable for participation in the study.

2.3 Treatment Regimen

Dose selection: Tislelizumab: 200mg. Pemetrexed: 500mg/m^2. Nab-paclitaxel: 260mg/m^2. Platinum drugs (Cisplatin: 60-80mg/m^2; Carboplatin: AUC=5; Nedaplatin: 80mg/m^2). Capsulized FMT: 30 capsules each time. Capsules will be stored in the refrigerator at -80° C. When taking capsules, take them out of the refrigerator, heat them in a water bath (single-hole water bath, JF-01 defroster) at 37 °C for 10 minutes, and swallow them with warm water.

Patients will receive the first-line regimen of Nab-paclitaxel + Platinum + Tislelizumab

(squamous cell lung cancer) / Pemetrexed + Platinum + Tislelizumab (lung adenocarcinoma) for 4-6 cycles. If there is no progression of the disease after 4-6 cycles of first-line treatment, then patients will enter the maintenance treatment stage. Patients will receive Tislelizumab maintenance treatment (squamous cell lung cancer), or Tislelizumab + Pemetrexed maintenance treatment (lung adenocarcinoma). Capsulized FMT should be conducted on the 5th day (±2 days) after the start of the 1, 3 and 5 cycles of chemotherapy, as well as on the 5th day (±2 days) after the first maintenance chemotherapy (lung adenocarcinoma) and before the first maintenance immunotherapy (squamous cell lung cancer). See Figures 2 for details

2.4 Study Endpoints

The primary endpoint of the study is the 12-month PFS rate. Secondary endpoints include ORR, mPFS, overall survival (OS), duration of response (DOR), safety, microbiome diversity, and quality of life (QoL). Exploratory endpoints including efficacy predictive biomarkers, including fungi, bacteria, metabolomics, and proteomics.

2.5 Sample Size Estimation

The parameters are set as follows: the two-sided significance level alpha is set at 0.05. Based on an estimated dropout rate of 0% and a reference 12-month PFS rate of 35% published in the Rational-304 and 307 clinical studies, it is inferred that the 12-month PFS rate in this trial can reach 55%.[2,3] Therefore, a total sample size of 62 participants is determined for this study to ensure statistical power greater than 80%.

2.6 Efficacy and Safety Assessments

Imaging evaluation

The assessments of the screening period should be conducted within 28 days prior to the first administration of the study drug. Prior to treatment, researchers at the study center will confirm that subjects have measurable lesions that met the RECIST 1.1 criteria. The methods used to assess tumor burden at baseline must be consistent with those used for each subsequent follow-up assessment (CT/MRI). Additional imaging assessments of

other suspected involved areas (eg, brain) may be conducted based on the subject's clinical symptoms and signs. After enrollment, tumor status will be evaluated using imaging methods every 6 weeks (±7 days), every 12 weeks (±7 days) after 48 weeks until disease progression (RECIST 1.1) or death (during the course of patient treatment). For subjects who have completed treatment or have discontinued treatment for reasons other than disease progression, a single tumor imaging assessment should be performed at the time of treatment completion/discontinuation.

Safety evaluation

Researchers will conduct safety assessments on patients every 3 weeks using NCI-CTCAE 5.0. After treatment ends, participants will be followed up for 30 days to detect adverse events (AE). If the patient has not received new anti-tumor treatment within 90 days after the last dose, severe adverse events (SAE) occurring within 90 days after the last dose will be collected. If the subject has received new anti-tumor treatment, SAE prior to the new treatment will be collected, with precedence given to those that have already occurred. Investigators should grade and record adverse events for each subject according to the NCI-CTCAE 5.0 criteria during the study and follow-up period. The characteristics of adverse events will be assessed and recorded based on severity, causality, toxicity grading, management measures, and outcomes.

Fecal and peripheral blood collection time

With the permission of the ethics committee, patients should provide 10ml whole blood samples and fecal samples at baseline, after two cycles of treatment, before maintenance treatment, and after two cycles of maintenance treatment for the detection of efficacy prediction markers (each cycle is 21 days).

2.7 Data Management

Data management methods

The study will use a data collection system (91trial) for data management. All subjects will be assigned a unique ID. Researchers will input basic information, drug use, laboratory examination and other raw data information of subjects into 91trial. The system is subject to superior monitoring and cannot be arbitrarily modified. The data manager will write a data audit report based on the trial protocol and audit criteria in the database. The results of the patient's report on tests, examinations, etc. can be obtained from the electronic case report. Quality of life will be assessed using the EORTC-QLQ-C30 questionnaire, which will be available in both paper and electronic formats.

Reporting and collection of adverse events

When a clinical adverse event occurs, it will be detailed on the case report form with the time of occurrence, clinical manifestations, course of treatment, duration, outcome, and the relationship to this study. For those with laboratory abnormalities, follow-up will continue until the test results return to normal, or to the level before medical intervention, or until it is determined that the event is unrelated to the medical intervention. In the event of a serious adverse event, the serious adverse event form should be filled out and reported to the department and relevant functional departments within 24 hours and recorded in the hospital quality control system (HQS) system, with documentation in the medical record.

2.8 Statistical Analysis

SPSS Statistic will be used for statistical processing in this study. Kaplan-Meier survival analysis will be used to draw PFS and OS survival curves, and Log-rank or Breslow test will be used to explore statistical significance. Multivariate analysis of PFS rate and OS will be performed by Cox proportional risk model.

2.9 Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or

dissemination plans of this research.

3. Ethics and Dissemination

This prospective study has been approved by the Clinical Medical Technology Ethics Committee of Changzhou No.2 People's Hospital (number [2024] YLJSA005) and has been registered in ClinicalTrials.gov (NCT06403111).

4. Discussion

The application of ICIs has brought significant benefits to patients with driver gene-negative NSCLC, but statistics show that the 5-year survival rate for advanced NSCLC patients receiving immunotherapy is only about 20%.[13] With a deeper exploration of the mechanisms of immune resistance, researchers have found that the gut microbiota plays a crucial role in regulating the immune system and can significantly influence the effectiveness of immunotherapy.

Preclinical trials have demonstrated that specific gut microbiota or fecal microbiota transplantation using feces from patients who respond to ICIs can modulate the immune system, enhance immune cell infiltration in tumors, induce tumor regression, and improve the antitumor efficacy of ICIs.[7,14–16] Existing clinical trials have shown that exogenous microflora supplementation by FMT can change the composition of intestinal microflora and the proportion of dominant microflora, reprogram the tumor microenvironment, and overcome immune resistance.[11,17] However, the majority of related studies have focused on melanoma, with limited research in the field of lung cancer treatment. A proof-of-concept clinical trial conducted by a team of researchers in Korea has demonstrated the potential benefits of FMT in a clinical setting other than melanoma, giving us confidence to carry out our study.[12] Therefore, we designed a multi-prospective, multicenter, single-arm exploratory study of patients with advanced NSCLC. If patients treated with FMT have longer PFS and higher 12-months PFS rate,

this study is expected to provide strong evidence for further improving outcomes in lung cancer patients.

Clinical Trial Status

At the time of submission, the study is ongoing and open to recruitment patients.

Authors' Contributions

YW and LQ are responsible for the article's concept and writing; QG, DL, and DQ are responsible for clinical treatment; QG, LQ, YW, and XW are responsible for data collection and compilation. HJ and QG coordinate all the work. All authors review and approve the final manuscript.

Funding

This study was supported by the 2022 Clinical Research project of Changzhou Medical Center, Nanjing Medical University (CMCC202201), 2022 Changzhou 8th Batch of Science and Technology Project (Applied Basic Research) (CJ20220086).

Competing interests

All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf and declare: no support from any organization for the submitted work; no financial relationships with any organization that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements

We would like to express our sincere appreciation to all the participants.

Peer review

Not externally peer reviewed.

Data sharing

No additional data are available.

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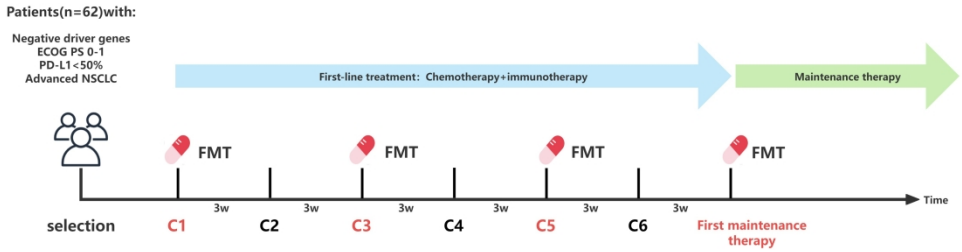

Figure 2 Timeline for FMT

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BMJ Open

Fecal microbiota transplantation combined with platinum-based doublet chemotherapy and Tislelizumab as first-line treatment for driver-gene negative advanced non-small-cell lung cancer (NSCLC): study protocol for a prospective, multi-center, single-arm exploratory trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-094366.R1
Article Type:	Protocol
Date Submitted by the Author:	20-Dec-2024
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Primary Subject Heading:	Oncology
Secondary Subject Heading:	Research methods
Keywords:	CHEMOTHERAPY, Clinical Relevance, Clinical Trial, ONCOLOGY, Respiratory tract tumours < ONCOLOGY

Fecal microbiota transplantation combined with platinum-based doublet chemotherapy and Tislelizumab as first-line treatment for driver-gene negative advanced non-small-cell lung cancer (NSCLC): study protocol for a prospective, multi-center, single-arm exploratory trial

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ABSTRACT

Introduction: Chemotherapy combined with immunotherapy is the standard first-line treatment for driver-gene negative advanced non-small cell lung cancer (NSCLC). However, due to the immune microenvironment imbalance and immune status impairment caused by repeated chemotherapy, as well as the primary or secondary resistance to immune checkpoint inhibitors (ICIs), the efficacy of immunotherapy combined with chemotherapy remains unsatisfactory. Recent studies have shown that fecal microbiota transplantation (FMT) can modulate the intestinal microflora, influence the tumor immune microenvironment, and even enhance the efficacy of immunotherapy. Therefore, we conduct this study to evaluate the efficacy and safety of FMT combined with standard first-line treatment for driver gene-negative advanced NSCLC.

Methods and analysis: FMT-JSNO-02 (NCT06403111) is a prospective, multi-center, single-arm exploratory study. It is planned to include 62 cases of previously untreated driver-gene negative, Eastern Cooperative Oncology Group Performance Status （ECOG PS) 0-1, Programmed death ligand 1 (PD-L1) <50% advanced NSCLC patients, who will be given FMT by orally ingested stool capsules on the basis of

standard first-line treatment of chemotherapy combined with immunotherapy. The safety and efficacy of this treatment regimen will be evaluated, with the primary endpoint being the 12-months progression-free survival (PFS) rate.

Ethics and dissemination: The study was approved by the ethics committee of the Second Peoples' Hospital of Changzhou (number [2024] YLJSA005) and is being conducted in accordance with the principles of the Declaration of Helsinki. The results of the study will be submitted for publication in peer-reviewed journals.

Trial registration number: ClinicalTrials.gov Identifier: NCT06403111. Date of registration: May 7, 2024, the first version protocol.

Strengths and limitations of this study:

- This is a multi-center prospective study.
- Our study aims to regulate intestinal flora and introduce FMT into the standard first-line treatment of lung cancer for the first time.
- The sample size in this study is relatively small.
- This is a single-arm study rather than a randomized controlled study.

keywords: Fecal microbiota transplantation, Non-Small Cell Lung Cancer, Chemotherapy, Immunotherapy

1. Introduction

Current Situation and Dilemmas of Immunotherapy for Non-Small Cell lung cancer (NSCLC)

Lung cancer is the most common malignancy worldwide and remains the leading cause of cancer-related mortality, accounting for 18.7% of all cancer deaths.[1]As the main pathological subtype of lung cancer, the majority of NSCLC patients present with local spread or distant metastasis upon diagnosis, losing the opportunity for surgery and having a very poor prognosis. In recent years, immune checkpoint inhibitors (ICIs), particularly those targeting the programmed death receptor 1 (PD-1) and its ligand (PD-L1), markedly improved the treatment outcomes and becoming an effective treatment strategy for NSCLC.

At present, the combination of chemotherapy and immunotherapy has become the standard first-line treatment for patients with advanced NSCLC due to extensive clinical studies conducted on pembrolizumab and nivolumab. Tislelizumab developed in China has also shown remarkable efficacy. In patients with lung adenocarcinoma,

the addition of tislelizumab to chemotherapy resulted in significantly prolonged median progression-free survival (mPFS) compared to chemotherapy alone (9.7 versus 7.6 month), with a 12-month PFS rate of 31.3%.[2] Similarly, in patients with lung squamous cell carcinoma, the mPFS for tislelizumab plus chemotherapy group was also higher than that of chemotherapy alone (9.6 months versus 5.5 months).[3]

Although ICIs significantly improved the survival of patients with advanced NSCLC, primary or acquired immune resistance inevitably occurs in clinical practice. Existing research suggests that primary immune resistance is related to the dysfunction in tumor antigen processing and presentation, insufficient T cell infiltration in the tumor microenvironment (TME), and over expression of suppressive immune cells.[4] Acquired immune resistances is related to adaptive changes that occur in tumor cells and the TME during exposure to immunotherapy.[5]

Nowadays, a growing body of research suggests that gut flora may be able to reverse immune resistance. For instance, gut microbiota can regulate the infiltration of immune cells in the TME by modulating innate and adaptive immunity. They can also reprogram the TME through their reactive metabolites or secretions, affecting the efficacy of ICIs and even reversing immune resistance.[6] Researchers have conducted preliminary explorations into how gut microbiota affects the efficacy of immunotherapy. Zitvoge found that the antitumor effects of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade depended on distinct Bacteroides species. Tumors in antibiotic-treated or germ-free mice did not respond to CTLA-4 blockade. This defect was overcome by gavage with B. fragilis, by immunization with B. fragilis polysaccharides, or by adoptive transfer of B. fragilis-specific T cells.[7] At the same time, Sivan et al. found that mice orally administered Bifidobacterium could achieve the same anti-tumor effect as PD-L1 monoclonal antibodies, and the combination almost completely inhibited tumor growth.[8] Therefore, modulating gut microbes has the potential to further improve the treatment outcomes for patients receiving immunotherapy.

Current Status of Fecal Microbiota Transplantation (FMT)

The gut microbiota is susceptible to various factors, including the use of broad-spectrum antibiotics, repeated chemotherapy and other treatments. Common methods to regulate the intestinal microbiota include dietary intervention, probiotics, prebiotics, and FMT. Among these approaches, FMT stands as the most established technique.[9] FMT is the transplantation of functional flora from the stool of a healthy person into the intestine of a patient in a certain way. This method mainly includes two ways: injecting microflora fluids and swallowing capsules. Among them, FMT by orally ingested stool capsules (capsulized FMT) has the characteristics of a wide range of application, high acceptance degree, relative economy and convenience.[10]

The relationship between intestinal flora and immunotherapy efficacy has been preliminarily confirmed in some clinical studies of malignant tumors. For example, in a study focused on 15 melanoma patients resistant to anti-PD-1 therapy, rechallenge with FMT and Pembrolizumab showed objective responses in 3 out of 15 patients, and these 3 patients kept stable disease (SD) for more than 12 months.[11] In a clinical trial (NCT04264975), researchers performed FMT with anti-PD-1 inhibitors in 13 patients with anti-PD-1 refractory advanced solid cancers. The results showed that FMT induced sustained microbiota changes and clinical benefits in 6 patients, achieving an objective response rate (ORR) of 7.7% and a disease control rate (DCR) of 46.2%.[12] As a result, FMT holds great potential to reverse immune resistance and enhance the efficacy of immunotherapy.

Hence, we conduct such a prospective, exploratory study in order to evaluate the efficacy and safety of FMT combined with standard first-line treatment for driver gene-negative advanced NSCLC. We expect that the addition of FMT to the standard first-line treatment in NSCLC will be safe and will have an enhancing effect.

2. Methods

2.1 Study Design

This is a prospective, multi-center, single-arm exploratory study aimed at evaluating the efficacy and safety of FMT combined with standard first-line treatment for driver-gene negative NSCLC patients. The trial protocol was written in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidance. The study will mainly be conducted at the Second Peoples' Hospital of Changzhou, the Third Affiliated Hospital of Nanjing Medical University. Recruitment information can be obtained through outpatient consultations, posters displayed in the wards, and other channels. A total of 62 patients with driver-gene negative, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-1, and PD-L1<50% who have not received prior treatment are expected to be included in this study without grouping. Patients will receive platinum-based doublet chemotherapy combined with tislelizumab as standard first-line treatment for 4-6 cycles and then enter maintenance therapy. FMT will be given to patients during the first, third, and fifth treatment cycles, as well as during the first maintenance therapy. All the enrolled patients or their designated agents will sign the informed consent form within 3 days before starting treatment. This study was approved by the ethics committee of the Second Peoples' Hospital of Changzhou (number [2024] YLJSA005). See figure 1 for details.

2.2 Patient Population

Inclusion criteria:

- The subjects voluntarily joined the study and were able to sign the informed consent with good compliance.
- Age 18-80 years old (when signing the informed consent form).
- Patients with histologically or cytologically proven locally advanced (ⅢB/ⅢC), metastatic, or recurrent (stage IV) NSCLC who are inoperable and unable to receive radical concurrent chemoradiotherapy, according to the International Association for the Study of Lung Cancer and the American Joint Committee on Cancer Classification, 8th Edition TNM Classification of Lung cancer.
- Have not received systemic intravenous anti-tumor therapy before, and the driver-gene is negative.
- PD-L1 expression < 50%.

- According to the solid tumor efficacy evaluation criteria (RECIST version 1.1), there is at least one radiographically measurable lesion. That is, in CT or MRI detection, the longest diameter of a single lesion was ≥ 10mm, or the pathological enlargement of a single lymph node was ≥ 15mm.
- The physical status score of Eastern Tumor Collaboration Group (ECOG) was 0-1.
- Expected survival > 3 months.
- Have adequate organ and bone marrow function, laboratory tests within 7 days prior to enrollment meet the following requirements (no blood components, cell growth factors, albumin or other corrective drugs are allowed within 14 days prior to obtaining laboratory examination), as follows: 1) Blood routine: absolute neutrophil count (ANC) ≥ 1.5×10^9/L, platelet (PLT) ≥ 75×10^9/L, hemoglobin (HGB) ≥ 90 g/L (no blood transfusion or erythropoietin dependence within 14 days). 2) Liver function: serum total bilirubin (TBIL) ≤ 2 times the upper limit of normal (ULN). Alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) ≤ 5×ULN, serum albumin ≥ 28 g/L. alkaline phosphatase (ALP) ≤ 5×ULN. 3) Renal function: serum creatinine (Cr) ≤ 1.5×ULN, or creatinine clearance ≥ 50 mL/min (using the standard Cockcroft-Gault formula): Urine routine results showed urinary protein < 2+. For patients with urine protein ≥ 2+ at baseline, 24-hour urine collection and 24-hour urine protein quantification < 1g should be performed. 4) Coagulation function: International standardized ratio (INR) or prothrombin time (PT) ≤ 1.5 times ULN. If the subject is receiving anticoagulant therapy, as long as the INR is within the intended range of anticoagulant drug use.
- For female subjects of reproductive age, a urine or serum pregnancy test should be performed and the result is negative 3 days prior to receiving the initial study drug administration.
- Subjects and their sexual partners are required to use a medically approved contraceptive method (such as an intrauterine device (IUD), contraceptive pill, or condom) during the study treatment period and for 6 months after the end of the study treatment period.

Exclusion criteria:

- Currently participating in an interventional clinical study or receiving another investigational drug or investigational device within 4 weeks prior to initial dosing.
- Received proprietary Chinese medicines with anti-tumor indications or immunomodulatory drugs (thymosin, interferon, interleukin, etc.) within 2 weeks before the first administration, or received major surgical treatment within 3 weeks before the first administration.
- Class III - IV congestive heart failure (New York Heart Association classification), poorly controlled and clinically significant arrhythmias.
- Any arterial thrombosis, embolism or ischemia, such as myocardial infarction, unstable angina pectoris, cerebrovascular accident or transient ischemic attack, occurred within 6 months before treatment.
- Known allergic reaction to the drug in this study.
- Patients who require long-term oral, intravenous, or intramuscular administration of systemic corticosteroids.
- Symptomatic central nervous metastases. Patients with asymptomatic brain metastases (BMS) or BMS whose symptoms are stable after treatment are eligible to participate in this study if they meet all of the following criteria: measurable lesions outside the central nervous system. No midbrain, pontine, cerebellum, meninges, medulla oblongata or spinal cord metastasis. Maintain clinical stability for at least 2 weeks. Stop hormone therapy 3 days before the first dose of the study drug.
- There is an active infection requiring treatment or systemic anti-infective drugs have been used in the week prior to the first dosing.
- Has not fully recovered from toxicity and/or complications caused by any intervention before starting treatment (i.e., ≤ grade 1 or baseline, excluding weakness or hair loss).
- Known history of human immunodeficiency virus (HIV) infection (i.e. HIV 1/2 antibody positive).
- Untreated active hepatitis B (defined as HBsAg positive and HBV-DNA copy

number detected greater than the upper limit of normal value in the laboratory of the study center).

- Active HCV-infected subjects (HCV antibody positive and HCV-RNA levels above the lower limit of detection).
- Received live vaccine within 30 days prior to the first dose (cycle 1, day 1). Note: Injectable inactivated virus vaccine against seasonal influenza is permitted for 30 days prior to initial administration. However, live attenuated influenza vaccines administered intranasally are not permitted.
- Pregnant or lactating women.
- Medical history or evidence of disease that may interfere with test results, prevent participants from fully participating in the study, abnormal treatment or laboratory test values, or other conditions that the investigator considers unsuitable for enrollment. The Investigator considers other potential risks unsuitable for participation in the study.

2.3 Treatment Regimen

Dose selection: tislelizumab: 200mg. pemetrexed: 500mg/m^2. albumin-bound paclitaxel: 260mg/m^2. platinum drugs (cisplatin: 60-80mg/m^2; carboplatin: AUC=5; nedaplatin: 80mg/m^2). Capsulized FMT: 30 capsules each time. Capsules will be stored in the refrigerator at -80°C. When taking capsules, take them out of the refrigerator, heat them in a water bath (single-hole water bath, JF-01 defroster) at 37°C for 10 minutes, and swallow them with warm water.

Participants will receive FMT combined with tislelizumab + pemetrexed + platinum-based treatment (lung adenocarcinoma) / albumin-bound paclitaxel + platinum-based treatment (lung squamous cell carcinoma) for 4-6 cycles. If there is no progression of the disease after 4-6 cycles of the first-line treatment, then patients will enter the maintenance treatment stage. Patients will receive tislelizumab maintenance treatment (lung squamous cell carcinoma), or tislelizumab + pemetrexed maintenance treatment (lung adenocarcinoma). Treatment continues until disease progression, subject withdraws informed consent, loss of follow-up, or death.

Capsulized FMT should be conducted on the 5th day (±2 days) after the start of the first, third, and fifth treatment cycles of chemotherapy, as well as on the 5th day (±2 days) after the first maintenance chemotherapy (lung adenocarcinoma) and before the first maintenance immunotherapy (lung squamous cell carcinoma). All patients will be prohibited from receiving any other treatments with anti-tumor activity or potential anti-tumor activity during the study. This will be monitored by the investigators, the patients, and their families. See figure 2 for details.

2.4 Study Endpoints

The primary endpoint of the study is the 12-month PFS rate. Secondary endpoints include ORR, mPFS, overall survival (OS), duration of response (DoR), safety, microbiome diversity, and quality of life (QoL). Exploratory endpoints including efficacy predictive biomarkers, including fungi, bacteria, metabolomics, and proteomics.

2.5 Sample Size Estimation

The parameters are set as follows: the two-sided significance level alpha is set at 0.05. Based on an estimated dropout rate of 0% and a reference 12-month PFS rate of 35% published in the RATIONALE-304 and RATIONALE-307 clinical studies, it is inferred that the 12-month PFS rate in this trial can reach 55%.[2,3] Therefore, a total sample size of 62 participants is determined for this study to ensure statistical power greater than 80%.

2.6 Efficacy and Safety Assessments

Imaging evaluation

The assessments of the screening period should be conducted within 28 days prior to the first administration of the study drug. Prior to treatment, researchers at the study center will confirm that subjects have measurable lesions that met the RECIST 1.1 criteria. The methods used to assess tumor burden at baseline must be consistent with those used for each subsequent follow-up assessment (CT/MRI). Additional imaging assessments of other suspected involved areas (e.g., brain) may be conducted based on the subject's clinical symptoms and signs. After enrollment, tumor status will be

evaluated using imaging methods every 6 weeks (± 7 days), every 12 weeks (± 7 days) after 48 weeks until disease progression (RECIST 1.1) or death (during the course of patient treatment). For subjects who have completed treatment or have discontinued treatment for reasons other than disease progression, a single tumor imaging assessment should be performed at the time of treatment completion/discontinuation.

Safety evaluation

Researchers will conduct safety assessments on patients every 3 weeks using NCI-CTCAE 5.0. After treatment ends, participants will be followed up for 30 days to detect adverse events (AE). If the patient has not received new anti-tumor treatment within 90 days after the last dose, severe adverse events (SAE) occurring within 90 days after the last dose will be collected. If the subject has received new anti-tumor treatment, SAE prior to the new treatment will be collected, with precedence given to those that have already occurred. Investigators should grade and record adverse events for each subject according to the NCI-CTCAE 5.0 criteria during the study and follow-up period. The characteristics of adverse events will be assessed and recorded based on severity, causality, toxicity grading, management measures, and outcomes.

Fecal and peripheral blood collection time

With the permission of the ethics committee, patients should provide 10ml whole blood samples and fecal samples at baseline, after two cycles of treatment, before maintenance treatment, and after two cycles of maintenance treatment for the detection of efficacy prediction markers (each cycle is 21 days).

2.7 Data Management

Data management methods

The study will use a data collection system (91trial) for data management. All subjects will be assigned a unique ID. Researchers will input basic information, drug use, laboratory examination and other raw data information of subjects into 91trial. The system is subject to superior monitoring and cannot be arbitrarily modified. The data manager will write a data audit report based on the trial protocol and audit criteria in the database. The results of the patient's report on tests, examinations, etc. can be

obtained from the electronic case report. Quality of life will be assessed using the EORTC-QLQ-C30 questionnaire, which will be available in both paper and electronic formats.

Throughout the data collection process, researchers will implement preventive measures to ensure the confidentiality of the documents and safeguard against the identification of participants. All data will be monitored by the data monitoring committee.

Reporting and collection of adverse events

When a clinical adverse event occurs, it will be detailed on the case report form with the time of occurrence, clinical manifestations, course of treatment, duration, outcome, and the relationship to this study. For those with laboratory abnormalities, follow-up will continue until the test results return to normal, or to the level before medical intervention, or until it is determined that the event is unrelated to the medical intervention. In the event of a serious adverse event, the serious adverse event form should be filled out and reported to the department and relevant functional departments within 24 hours and recorded in the hospital quality control system (HQS) system, with documentation in the medical record.

2.8 Statistical Analysis

This study will use SPSS statistical software for data analysis. By recording patients' survival times, Kaplan-Meier survival curves for PFS%, PFS and OS will be plotted to visually display the trend of survival rates over time. The occurrence of AE and SAE will be recorded, and the frequency and percentage of different levels of adverse events will be used to describe their occurrence. The Log-rank test will be used to compare whether there are statistically significant differences between the historical control group and the experimental group.

Subgroups will be divided based on patients' demographic characteristics (age, sex), tobacco use, nutritional status, ECOG performance status, pathological features (tumor type, solid tumor stage, pathological subtype), metastatic sites (such as brain metastasis, liver metastasis), PD-L1 expression, and other relevant factors. A multivariate analysis

of PFS and OS will be conducted using the Cox proportional hazards model. A p-value of <0.05 will be considered statistically significant.

2.9 Trial status

The study was registered at ClinicalTrials.gov (NCT06403111) on May 7, 2024. Enrolment is currently in progress. The first patient was enrolled on June 25, 2024, and the study is expected to end on June 1, 2026.

2.10 Patient and public involvement

None.

3. Ethics and Dissemination

The study was approved by the ethics committee of the Second Peoples' Hospital of Changzhou (number [2024] YLJSA005) and is being conducted in accordance with the principles of the Declaration of Helsinki. The study was registered in ClinicalTrials.gov (NCT06403111). Important protocol modifications will be communicated to relevant parties and published on ClinicalTrials.gov. Before participating in the study, the participants (or their legal representatives) will sign the informed consent form. During the study, participants will be provided with any new information that may affect their decision to continue. They can withdraw at any time without facing any penalties or losing any benefits to which they are entitled. The results of this study will be disseminated in a peer-reviewed journal and in conference reports.

4. Discussion

The application of ICIs has brought significant benefits to patients with driver gene-negative NSCLC, but statistics show that the 5-year survival rate for advanced NSCLC patients receiving immunotherapy is only about 20%.[13] With a deeper exploration of the mechanisms of immune resistance, researchers have found that the gut microbiota plays a crucial role in regulating the immune system.

Preclinical studies have found that specific gut microbiota or fecal microbiota transplantation using feces from patients who respond to ICIs can modulate the immune

system, enhance immune cell infiltration in tumors, induce tumor regression, and improve the antitumor efficacy of ICIs.[8,14–16] Existing clinical trials have shown that exogenous microflora supplementation by FMT can change the composition of intestinal microflora and the proportion of dominant microflora, reprogram the tumor microenvironment, and potentially reverse immune resistance.[11,17] However, the majority of related studies have focused on melanoma, with limited research in the field of lung cancer treatment. Recently, a proof-of-concept clinical trial conducted by a team of researchers in Korea has demonstrated the potential benefits of FMT in a clinical setting other than melanoma, giving us confidence to carry out our study.[12]

Our study is a single-arm clinical trial, which has the limitations of a small sample size and the absence of a control group. So, the conclusions may not be convincing. At the same time, some information derived from a single-arm trial, such as QoL and efficacy predictive biomarkers was relatively limited. Though the results of randomized controlled trials are more convincing, it demands significant investments of human, material, and financial resources.[18] On this basis, randomized phase II trials seem to be an effective research method, which requires a relatively small sample size compared to traditional randomized controlled trials, and the inclusion of a control group makes it more scientifically rigorous than a single-arm study.[19] Overall, each research method has its own strengths and disadvantages. Therefore, we should consider it carefully in the light of the actual situation.

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Authors' Contributions

YW and LQ are responsible for the article's concept and writing. QG, DL, and DQ are responsible for clinical treatment. QG, LQ, YW, and XW are responsible for data collection and compilation. HJ and QG coordinate all the work. YW and LQ contributed equally to this paper. QG is the guarantor, reviews the entire study design and the draft. All authors review and approve the final manuscript.

Funding

This study was supported by the 2022 Clinical Research project of Changzhou Medical Center, Nanjing Medical University (CMCC202201), 2022 Changzhou 8th Batch of Science and Technology Project (Applied Basic Research) (CJ20220086).

Acknowledgements

We would like to express our sincere appreciation to all the participants.

Peer review

Not externally peer reviewed.

Data sharing

No additional data are available.

Competing interests statement

There are no associations with commercial entities, nor any financial relationships involving their spouse or children under 18 years of age. Additionally, there are no non-financial associations that could be relevant to the submitted manuscript.

Figure legends

figure 1 The participant pathway of the clinical trial

figure 2 Timeline for FMT

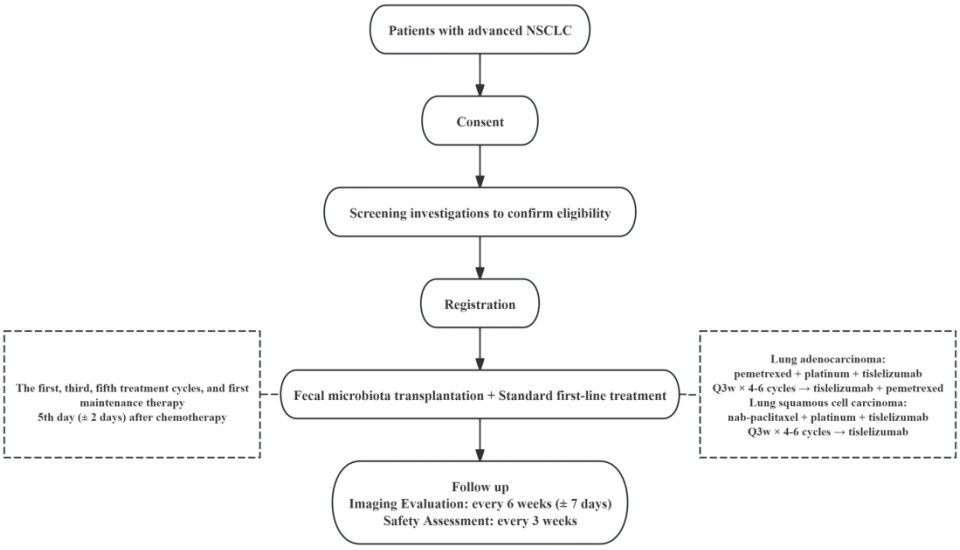

figure 1 The participant pathway of the clinical trial

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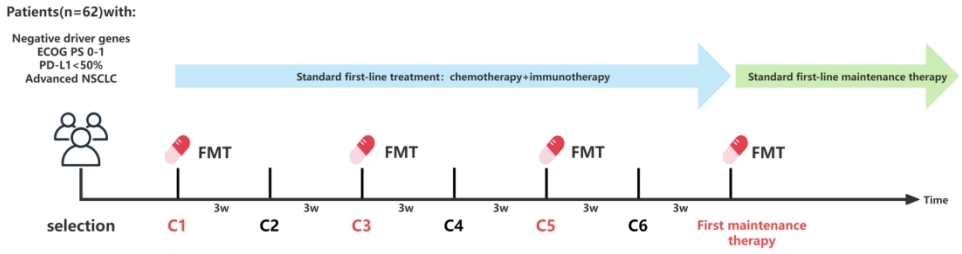

figure 2 Timeline for FMT

171x52mm (300 x 300 DPI)

BMJ Open

Fecal microbiota transplantation combined with platinum-based doublet chemotherapy and Tislelizumab as first-line treatment for driver-gene negative advanced non-small-cell lung cancer (NSCLC): study protocol for a prospective, multi-center, single-arm exploratory trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-094366.R2
Article Type:	Protocol
Date Submitted by the Author:	27-Jan-2025
Complete List of Authors:	Wei, Yanshuang; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology Qin, Lanqun; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology; Nanjing Medical University, Changzhou Medical Center Wu, Xinyu; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology Li, Dongqing; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology; Nanjing Medical University, Changzhou Medical Center Qian, Danping; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology; Nanjing Medical University, Changzhou Medical Center Jiang, Hua; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology; Nanjing Medical University, Changzhou Medical center Geng, Qian; Changzhou Second People's Hospital of Nanjing Medical University, Department of Oncology; Nanjing Medical University, Changzhou Medical Center
Primary Subject Heading:	Oncology
Secondary Subject Heading:	Research methods
Keywords:	CHEMOTHERAPY, Clinical Relevance, Clinical Trial, ONCOLOGY, Respiratory tract tumours < ONCOLOGY

Fecal microbiota transplantation combined with platinum-based doublet chemotherapy and Tislelizumab as first-line treatment for driver-gene negative advanced non-small cell lung cancer (NSCLC): study protocol for a prospective, multi-center, single-arm exploratory trial

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ABSTRACT

Introduction: Chemotherapy combined with immunotherapy is the standard first-line treatment for driver-gene negative advanced non-small cell lung cancer (NSCLC). However, due to the immune microenvironment imbalance and immune status impairment caused by repeated chemotherapy, as well as the primary or secondary resistance to immune checkpoint inhibitors (ICIs), the efficacy of immunotherapy combined with chemotherapy remains unsatisfactory. Recent studies have shown that fecal microbiota transplantation (FMT) can modulate the intestinal microflora, influence the tumor immune microenvironment, and even enhance the efficacy of immunotherapy. Therefore, we conduct this study to evaluate the efficacy and safety of FMT combined with standard first-line treatment for driver gene-negative advanced NSCLC.

Methods and analysis: FMT-JSNO-02 (NCT06403111) is a prospective, multi-center, single-arm exploratory study. It is planned to include 62 cases of previously untreated driver-gene negative, Eastern Cooperative Oncology Group Performance Status （ECOG PS) 0-1, Programmed death ligand 1 (PD-L1) <50% advanced NSCLC patients, who will be given FMT by orally ingested stool capsules on the basis of

standard first-line treatment of chemotherapy combined with immunotherapy. The safety and efficacy of this treatment regimen will be evaluated, with the primary endpoint being the 12-months progression-free survival (PFS) rate.

Ethics and dissemination: The study was approved by the ethics committee of the Second People's Hospital of Changzhou (number [2024] YLJSA005) and is being conducted in accordance with the principles of the Declaration of Helsinki. The results of the study will be submitted for publication in peer-reviewed journals.

Trial registration number: ClinicalTrials.gov Identifier: NCT06403111. Date of registration: May 7, 2024, the first version protocol.

Strengths and limitations of this study:

- This is a multi-center prospective study.
- Patients receive FMT via orally ingested stool capsules.
- The sample size in this study is relatively small.
- This is a single-arm study rather than a randomized controlled study.

keywords: Fecal microbiota transplantation, Non-Small Cell Lung Cancer, Chemotherapy, Immunotherapy

1. Introduction

Current Situation and Dilemmas of Immunotherapy for Non-Small Cell lung cancer (NSCLC)

Lung cancer is the most common malignancy worldwide and remains the leading cause of cancer-related mortality, accounting for 18.7% of all cancer deaths.[1]As the main pathological subtype of lung cancer, the majority of NSCLC patients present with local spread or distant metastasis upon diagnosis, losing the opportunity for surgery and having a very poor prognosis. In recent years, immune checkpoint inhibitors (ICIs), particularly those targeting the programmed death receptor 1 (PD-1) and its ligand (PD-L1), markedly improved the treatment outcomes and becoming an effective treatment strategy for NSCLC.

At present, the combination of chemotherapy and immunotherapy has become the standard first-line treatment for patients with advanced NSCLC due to extensive clinical studies conducted on pembrolizumab and nivolumab. Tislelizumab developed in China has also shown remarkable efficacy. In patients with lung adenocarcinoma, the addition of tislelizumab to chemotherapy resulted in significantly prolonged median

progression-free survival (mPFS) compared to chemotherapy alone (9.7 versus 7.6 month), with a 12-month PFS rate of 31.3%.[2] Similarly, in patients with lung squamous cell carcinoma, the mPFS for tislelizumab plus chemotherapy group was also higher than that of chemotherapy alone (9.6 months versus 5.5 months).[3]

Although ICIs significantly improved the survival of patients with advanced NSCLC, primary or acquired immune resistance inevitably occurs in clinical practice. Existing research suggests that primary immune resistance is related to the dysfunction in tumor antigen processing and presentation, insufficient T cell infiltration in the tumor microenvironment (TME), and over expression of suppressive immune cells.[4] Acquired immune resistances is related to adaptive changes that occur in tumor cells and the TME during exposure to immunotherapy.[5]

Nowadays, a growing body of research suggests that gut flora may be able to reverse immune resistance. For instance, gut microbiota can regulate the infiltration of immune cells in the TME by modulating innate and adaptive immunity. They can also reprogram the TME through their reactive metabolites or secretions, affecting the efficacy of ICIs and even reversing immune resistance.[6] Researchers have conducted preliminary explorations into how gut microbiota affects the efficacy of immunotherapy. Zitvoge found that the antitumor effects of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade depended on distinct Bacteroides species. Tumors in antibiotic-treated or germ-free mice did not respond to CTLA-4 blockade. This defect was overcome by gavage with B. fragilis, by immunization with B. fragilis polysaccharides, or by adoptive transfer of B. fragilis-specific T cells.[7] At the same time, Sivan et al. found that mice orally administered Bifidobacterium could achieve the same anti-tumor effect as PD-L1 monoclonal antibodies, and the combination almost completely inhibited tumor growth.[8] Therefore, modulating gut microbes has the potential to further improve the treatment outcomes for patients receiving immunotherapy.

Current Status of Fecal Microbiota Transplantation (FMT)

The gut microbiota is susceptible to various factors, including the use of broad-spectrum antibiotics, repeated chemotherapy and other treatments. Common methods to regulate the intestinal microbiota include dietary intervention, probiotics, prebiotics, and FMT. Among these approaches, FMT stands as the most established technique.[9] FMT is the transplantation of functional flora from the stool of a healthy person into the intestine of a patient in a certain way. This method mainly includes two ways: injecting microflora fluids and swallowing capsules. Among them, FMT by orally ingested stool capsules (capsulized FMT) has the characteristics of a wide range of application, high acceptance degree, relative economy and convenience.[10]

The relationship between intestinal flora and immunotherapy efficacy has been preliminarily confirmed in some clinical studies of malignant tumors. For example, in a study focused on 15 melanoma patients resistant to anti-PD-1 therapy, rechallenge with FMT and Pembrolizumab showed objective responses in 3 out of 15 patients, and these 3 patients kept stable disease (SD) for more than 12 months.[11] In a clinical trial (NCT04264975), researchers performed FMT with anti-PD-1 inhibitors in 13 patients with anti-PD-1 refractory advanced solid cancers. The results showed that FMT induced sustained microbiota changes and clinical benefits in 6 patients, achieving an objective response rate (ORR) of 7.7% and a disease control rate (DCR) of 46.2%.[12] As a result, FMT holds great potential to reverse immune resistance and enhance the efficacy of immunotherapy.

Hence, we conduct such a prospective, exploratory study in order to evaluate the efficacy and safety of FMT combined with standard first-line treatment for driver gene-negative advanced NSCLC. We expect that the addition of FMT to the standard first-line treatment in NSCLC will be safe and will have an enhancing effect.

2. Methods

2.1 Study Design

This is a prospective, multi-center, single-arm exploratory study aimed at evaluating

the efficacy and safety of FMT combined with standard first-line treatment for driver-gene negative NSCLC patients. The trial protocol was written in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidance. The study will mainly be conducted at the Second People's Hospital of Changzhou, the Third Affiliated Hospital of Nanjing Medical University. Recruitment information can be obtained through outpatient consultations, posters displayed in the wards, and other channels. A total of 62 patients with driver-gene negative, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-1, and PD-L1<50% who have not received prior treatment are expected to be included in this study without grouping. Patients will receive platinum-based doublet chemotherapy combined with tislelizumab as standard first-line treatment for 4-6 cycles and then enter maintenance therapy. FMT will be given to patients during the first, third, and fifth treatment cycles, as well as during the first maintenance therapy. All the enrolled patients or their designated agents will sign the informed consent form within 3 days before starting treatment. This study was approved by the ethics committee of the Second People's Hospital of Changzhou (number [2024] YLJSA005). See figure 1 for details.

2.2 Patient Population

Inclusion criteria:

- The subjects voluntarily joined the study and were able to sign the informed consent with good compliance.
- Age 18-80 years old (when signing the informed consent form).
- Patients with histologically or cytologically proven locally advanced (ⅢB/ⅢC), metastatic, or recurrent (stage IV) NSCLC who are inoperable and unable to receive radical concurrent chemoradiotherapy, according to the International Association for the Study of Lung Cancer and the American Joint Committee on Cancer Classification, 8th Edition TNM Classification of Lung cancer.
- Have not received systemic intravenous anti-tumor therapy before, and the driver-gene is negative.
- PD-L1 expression < 50%.
- According to the solid tumor efficacy evaluation criteria (RECIST version 1.1),

there is at least one radiographically measurable lesion. That is, in CT or MRI detection, the longest diameter of a single lesion was ≥ 10mm, or the pathological enlargement of a single lymph node was ≥ 15mm.

- The physical status score of Eastern Tumor Collaboration Group (ECOG) was 0-1.
- Expected survival > 3 months.
- Have adequate organ and bone marrow function, laboratory tests within 7 days prior to enrollment meet the following requirements (no blood components, cell growth factors, albumin or other corrective drugs are allowed within 14 days prior to obtaining laboratory examination), as follows: 1) Blood routine: absolute neutrophil count (ANC) ≥ 1.5×10^9/L, platelet (PLT) ≥ 75×10^9/L, hemoglobin (HGB) ≥ 90 g/L (no blood transfusion or erythropoietin dependence within 14 days). 2) Liver function: serum total bilirubin (TBIL) ≤ 2 times the upper limit of normal (ULN). Alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) ≤ 5×ULN, serum albumin ≥ 28 g/L. alkaline phosphatase (ALP) ≤ 5×ULN. 3) Renal function: serum creatinine (Cr) ≤ 1.5×ULN, or creatinine clearance ≥ 50 mL/min (using the standard Cockcroft-Gault formula): Urine routine results showed urinary protein < 2+. For patients with urine protein ≥ 2+ at baseline, 24-hour urine collection and 24-hour urine protein quantification < 1g should be performed. 4) Coagulation function: International standardized ratio (INR) or prothrombin time (PT) ≤ 1.5 times ULN. If the subject is receiving anticoagulant therapy, as long as the INR is within the intended range of anticoagulant drug use.
- For female subjects of reproductive age, a urine or serum pregnancy test should be performed and the result is negative 3 days prior to receiving the initial study drug administration.
- Subjects and their sexual partners are required to use a medically approved contraceptive method (such as an intrauterine device (IUD), contraceptive pill, or condom) during the study treatment period and for 6 months after the end of the study treatment period.

Exclusion criteria:

- Currently participating in an interventional clinical study or receiving another investigational drug or investigational device within 4 weeks prior to initial dosing.
- Received proprietary Chinese medicines with anti-tumor indications or immunomodulatory drugs (thymosin, interferon, interleukin, etc.) within 2 weeks before the first administration, or received major surgical treatment within 3 weeks before the first administration.
- Class III - IV congestive heart failure (New York Heart Association classification), poorly controlled and clinically significant arrhythmias.
- Any arterial thrombosis, embolism or ischemia, such as myocardial infarction, unstable angina pectoris, cerebrovascular accident or transient ischemic attack, occurred within 6 months before treatment.
- Known allergic reaction to the drug in this study.
- Patients who require long-term oral, intravenous, or intramuscular administration of systemic corticosteroids.
- Symptomatic central nervous metastases. Patients with asymptomatic brain metastases (BMS) or BMS whose symptoms are stable after treatment are eligible to participate in this study if they meet all of the following criteria: measurable lesions outside the central nervous system. No midbrain, pontine, cerebellum, meninges, medulla oblongata or spinal cord metastasis. Maintain clinical stability for at least 2 weeks. Stop hormone therapy 3 days before the first dose of the study drug.
- There is an active infection requiring treatment or systemic anti-infective drugs have been used in the week prior to the first dosing.
- Has not fully recovered from toxicity and/or complications caused by any intervention before starting treatment (i.e., ≤ grade 1 or baseline, excluding weakness or hair loss).
- Known history of human immunodeficiency virus (HIV) infection (i.e. HIV 1/2 antibody positive).
- Untreated active hepatitis B (defined as HBsAg positive and HBV-DNA copy

number detected greater than the upper limit of normal value in the laboratory of the study center).

- Active HCV-infected subjects (HCV antibody positive and HCV-RNA levels above the lower limit of detection).
- Received live vaccine within 30 days prior to the first dose (cycle 1, day 1). Note: Injectable inactivated virus vaccine against seasonal influenza is permitted for 30 days prior to initial administration. However, live attenuated influenza vaccines administered intranasally are not permitted.
- Pregnant or lactating women.
- Medical history or evidence of disease that may interfere with test results, prevent participants from fully participating in the study, abnormal treatment or laboratory test values, or other conditions that the investigator considers unsuitable for enrollment. The Investigator considers other potential risks unsuitable for participation in the study.

2.3 Treatment Regimen

Dose selection: tislelizumab: 200mg. pemetrexed: 500mg/m^2. albumin-bound paclitaxel: 260mg/m^2. platinum drugs (cisplatin: 60-80mg/m^2; carboplatin: AUC=5; nedaplatin: 80mg/m^2). Capsulized FMT: 30 capsules each time. Capsules will be stored in the refrigerator at -80°C. When taking capsules, take them out of the refrigerator, heat them in a water bath (single-hole water bath, JF-01 defroster) at 37°C for 10 minutes, and swallow them with warm water.

Participants will receive FMT combined with tislelizumab + pemetrexed + platinum-based treatment (lung adenocarcinoma) / albumin-bound paclitaxel + platinum-based treatment (lung squamous cell carcinoma) for 4-6 cycles. If there is no progression of the disease after 4-6 cycles of the first-line treatment, then patients will enter the maintenance treatment stage. Patients will receive tislelizumab maintenance treatment (lung squamous cell carcinoma), or tislelizumab + pemetrexed maintenance treatment (lung adenocarcinoma). Treatment continues until disease progression, subject withdraws informed consent, loss of follow-up, or death.

Capsulized FMT should be conducted on the 5th day (±2 days) after the start of the first, third, and fifth treatment cycles of chemotherapy, as well as on the 5th day (±2 days) after the first maintenance chemotherapy (lung adenocarcinoma) and before the first maintenance immunotherapy (lung squamous cell carcinoma). All patients will be prohibited from receiving any other treatments with anti-tumor activity or potential anti-tumor activity during the study. This will be monitored by the investigators, the patients, and their families. See figure 2 for details.

2.4 Study Endpoints

The primary endpoint of the study is the 12-month PFS rate. Secondary endpoints include ORR, mPFS, overall survival (OS), duration of response (DoR), safety, microbiome diversity, and quality of life (QoL). Exploratory endpoints including efficacy predictive biomarkers, including fungi, bacteria, metabolomics, and proteomics.

2.5 Sample Size Estimation

The parameters are set as follows: the two-sided significance level alpha is set at 0.05. Based on an estimated dropout rate of 0% and a reference 12-month PFS rate of 35% published in the RATIONALE-304 and RATIONALE-307 clinical studies, it is inferred that the 12-month PFS rate in this trial can reach 55%.[2,3] Therefore, a total sample size of 62 participants is determined for this study to ensure statistical power greater than 80%.

2.6 Efficacy and Safety Assessments

Imaging evaluation

The assessments of the screening period should be conducted within 28 days prior to the first administration of the study drug. Prior to treatment, researchers at the study center will confirm that subjects have measurable lesions that met the RECIST version 1.1 criteria. The methods used to assess tumor burden at baseline must be consistent with those used for each subsequent follow-up assessment (CT/MRI). Additional imaging assessments of other suspected involved areas (e.g., brain) may be conducted based on the subjects' clinical symptoms and signs. After enrollment, tumor status will

be evaluated using imaging methods every 6 weeks (± 7 days), every 12 weeks (± 7 days) after 48 weeks until disease progression (RECIST version 1.1) or death (during the course of patient treatment). For subjects who have completed treatment or have discontinued treatment for reasons other than disease progression, a single tumor imaging assessment should be performed at the time of treatment completion/discontinuation.

Safety evaluation

Researchers will conduct safety assessments on patients every 3 weeks using NCI-CTCAE 5.0. After treatment ends, participants will be followed up for 30 days to detect adverse events (AE). If the patient has not received new anti-tumor treatment within 90 days after the last dose, severe adverse events (SAE) occurring within 90 days after the last dose will be collected. If the subject has received new anti-tumor treatment, SAE prior to the new treatment will be collected, with precedence given to those that have already occurred. Investigators should grade and record adverse events for each subject according to the NCI-CTCAE 5.0 criteria during the study and follow-up period. The characteristics of adverse events will be assessed and recorded based on severity, causality, toxicity grading, management measures, and outcomes.

Fecal and peripheral blood collection time

With the permission of the ethics committee, patients should provide 10ml whole blood samples and fecal samples at baseline, after two cycles of treatment, before maintenance treatment, and after two cycles of maintenance treatment for the detection of efficacy prediction markers (each cycle is 21 days).

2.7 Data Management

Data management methods

The study will use a data collection system (91trial) for data management. All subjects will be assigned a unique ID. Researchers will input basic information, drug use, laboratory examination and other raw data information of subjects into 91trial. The system is subject to superior monitoring and cannot be arbitrarily modified. The data manager will write a data audit report based on the trial protocol and audit criteria in

the database. The results of the patients' report on tests, examinations, etc. can be obtained from the electronic case report. Quality of life will be assessed using the EORTC-QLQ-C30 questionnaire, which will be available in both paper and electronic formats.

Throughout the data collection process, researchers will implement preventive measures to ensure the confidentiality of the documents and safeguard against the identification of participants. All data will be monitored by the data monitoring committee.

Reporting and collection of adverse events

When a clinical adverse event occurs, it will be detailed on the case report form with the time of occurrence, clinical manifestations, course of treatment, duration, outcome, and the relationship to this study. For those with laboratory abnormalities, follow-up will continue until the test results return to normal, or to the level before medical intervention, or until it is determined that the event is unrelated to the medical intervention. In the event of a serious adverse event, the serious adverse event form should be filled out and reported to the department and relevant functional departments within 24 hours and recorded in the hospital quality control system (HQS) system, with documentation in the medical record.

2.8 Statistical Analysis

This study will use SPSS statistical software for data analysis. By recording patients' survival times, Kaplan-Meier survival curves for PFS%, PFS and OS will be plotted to visually display the trend of survival rates over time. The occurrence of AE and SAE will be recorded, and the frequency and percentage of different levels of adverse events will be used to describe their occurrence. The Log-rank test will be used to compare whether there are statistically significant differences between the historical control group and the experimental group.

Subgroups will be divided based on patients' demographic characteristics (age, sex), tobacco use, nutritional status, ECOG performance status, pathological features (tumor type, solid tumor stage, pathological subtype), metastatic sites (such as brain metastasis,

liver metastasis), PD-L1 expression, and other relevant factors. A multivariate analysis of PFS and OS will be conducted using the Cox proportional hazards model. A p-value of <0.05 will be considered statistically significant.

2.9 Trial status

The study was registered at ClinicalTrials.gov (NCT06403111) on May 7, 2024. Enrolment is currently in progress. The first patient was enrolled on June 25, 2024, and the study is expected to end on June 1, 2026.

2.10 Patient and public involvement

None.

3. Ethics and Dissemination

The study was approved by the ethics committee of the Second People's Hospital of Changzhou (number [2024] YLJSA005) and is being conducted in accordance with the principles of the Declaration of Helsinki. The study was registered in ClinicalTrials.gov (NCT06403111). Important protocol modifications will be communicated to relevant parties and published on ClinicalTrials.gov. Before participating in the study, the participants (or their legal representatives) will sign the informed consent form. During the study, participants will be provided with any new information that may affect their decision to continue. They can withdraw at any time without facing any penalties or losing any benefits to which they are entitled. If participants suffer harm due to research interventions or research-related procedures during the trial, appropriate compensation will be provided in accordance with relevant laws, regulations, and the guidance of the ethics committee. The results of this study will be disseminated in a peer-reviewed journal and in conference reports.

4. Discussion

The application of ICIs has brought significant benefits to patients with driver gene-negative NSCLC, but statistics show that the 5-year survival rate for advanced NSCLC patients receiving immunotherapy is only about 20%.[13] With a deeper exploration of

the mechanisms of immune resistance, researchers have found that the gut microbiota plays a crucial role in regulating the immune system.

Preclinical studies have found that specific gut microbiota or fecal microbiota transplantation using feces from patients who respond to ICIs can modulate the immune system, enhance immune cell infiltration in tumors, induce tumor regression, and improve the antitumor efficacy of ICIs.[8,14–16] Existing clinical trials have shown that exogenous microflora supplementation by FMT can change the composition of intestinal microflora and the proportion of dominant microflora, reprogram the tumor microenvironment, and potentially reverse immune resistance.[11,17] However, the majority of related studies have focused on melanoma, with limited research in the field of lung cancer treatment. Recently, a proof-of-concept clinical trial conducted by a team of researchers in Korea has demonstrated the potential benefits of FMT in a clinical setting other than melanoma, giving us confidence to carry out our study.[12]

Our study is a single-arm clinical trial, which has the limitations of a small sample size and the absence of a control group. So, the conclusions may not be convincing. At the same time, some information derived from a single-arm trial, such as QoL and efficacy predictive biomarkers was relatively limited. Though the results of randomized controlled trials are more convincing, it demands significant investments of human, material, and financial resources.[18] On this basis, randomized phase II trials seem to be an effective research method, which requires a relatively small sample size compared to traditional randomized controlled trials, and the inclusion of a control group makes it more scientifically rigorous than a single-arm study.[19] Overall, each research method has its own strengths and disadvantages. Therefore, we should consider it carefully in the light of the actual situation.

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Authors' Contributions

YW and LQ are responsible for the article's concept and writing. QG, DL, and DQ are responsible for clinical treatment. QG, LQ, YW, and XW are responsible for data collection and compilation. HJ and QG coordinate all the work. YW and LQ contributed equally to this paper. QG is the guarantor, reviews the entire study design and the draft. All authors review and approve the final manuscript.

Funding

This study was supported by the 2022 Clinical Research project of Changzhou Medical Center, Nanjing Medical University (CMCC202201), 2022 Changzhou 8th Batch of Science and Technology Project (Applied Basic Research) (CJ20220086).

Acknowledgements

We would like to express our sincere appreciation to all the participants.

Peer review

Not externally peer reviewed.

Data sharing

No additional data are available.

Competing interests

Not applicable.

Figure legends

figure 1 The participant pathway of the clinical trial

figure 2 Timeline for FMT

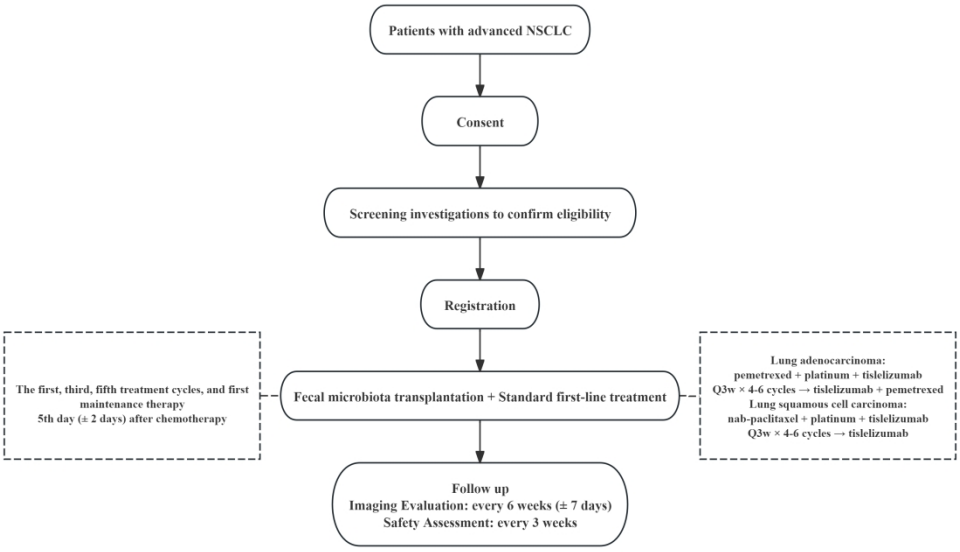

figure 1 The participant pathway of the clinical trial

170x100mm (462 x 462 DPI)

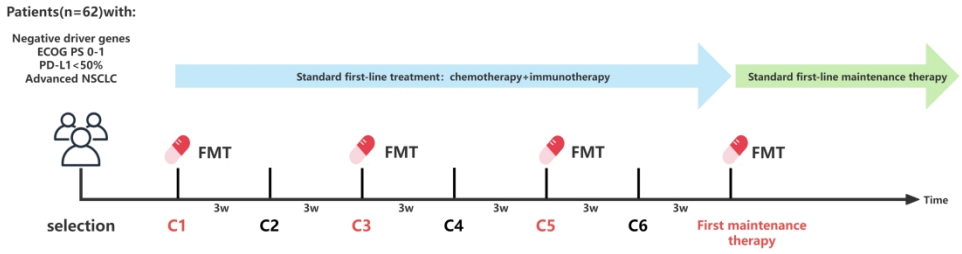

figure 2 Timeline for FMT

172x52mm (579 x 579 DPI)