
First Affiliated Hospital of Guangdong Pharmaceutical University

**Therapeutic effects of Compound Zhenzhu Tiaozhi capsules in nonalcoholic fatty liver
disease: a randomized controlled study**

Statistical proposal of treatment-naive patients

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1. Overview

This statistical proposal was established according to the clinical research study “Therapeutic effects of Compound Zhenzhu Tiaozhi capsules in nonalcoholic fatty liver disease: a randomized controlled study”, which will be conducted at the First Affiliated Hospital of Guangdong Pharmaceutical University. We declare that this protocol is consistent with the research project. This use of this protocol, including the research project and case report form, will start from November 11, 2017. Any further changes need to update SAP.

2. Objectives

2.1 Overall Objective

We aim to explore the safety and efficacy of Compound Zhenzhu Tiaozhi capsules in the treatment of non-alcoholic fatty liver disease (NAFLD) through a prospective, open-label, and randomized clinical study. We also expect to prevent the progression of simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), and expect to improve the efficacy of NAFLD treatment, while reducing the incidence of liver cirrhosis and hepatocellular carcinoma. We will then explore the mechanism of action of Compound Zhenzhu Tiaozhi capsules for the treatment of NAFLD.

2.2 Primary Objectives

1. To explore the safety and efficacy of Compound Zhenzhu Tiaozhi capsules for the treatment of NAFLD, compared with metformin and simvastatin.
2. To summarize the regularity of disease progression in the development of NAFLD.
3. To promote the therapeutic options of NAFLD and to improve long-term efficacy.
4. To explore the potential mechanism of Compound Zhenzhu Tiaozhi capsules for the treatment of NAFLD.

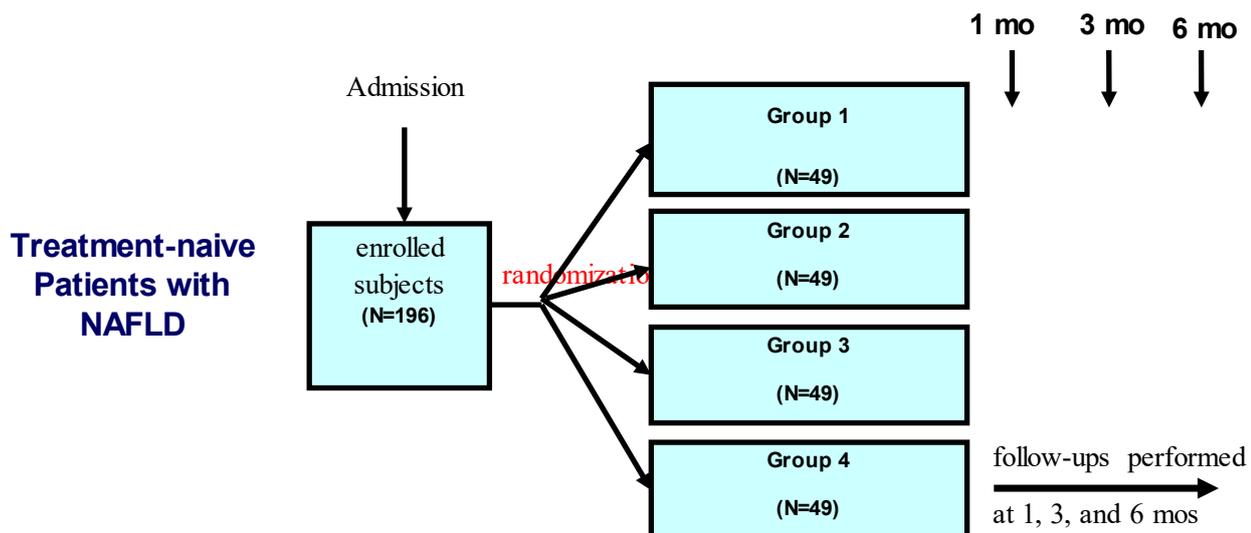
2.3 Secondary Objectives

1. To evaluate the change in intestinal mucosal barrier function by detecting biochemical parameters of the intestinal mucosal barrier; to analyze the relationship between NAFLD and the function of the intestinal mucosal barrier.
2. To explore the trends of small intestinal bacterial overgrowth (SIBO) during the treatment process.
3. To investigate whether lipid profiles and liver biochemical parameters are improved following treatment with Compound Zhenzhu Tiaozhi capsules.
4. To assess whether insulin resistance is improved following treatment with Compound Zhenzhu Tiaozhi capsules.
5. To monitor adverse events and put forward specific solutions for the adverse events.

3. Design, evaluation, and plans

3.1. Design

This is a prospective, open-label and randomized clinical study.

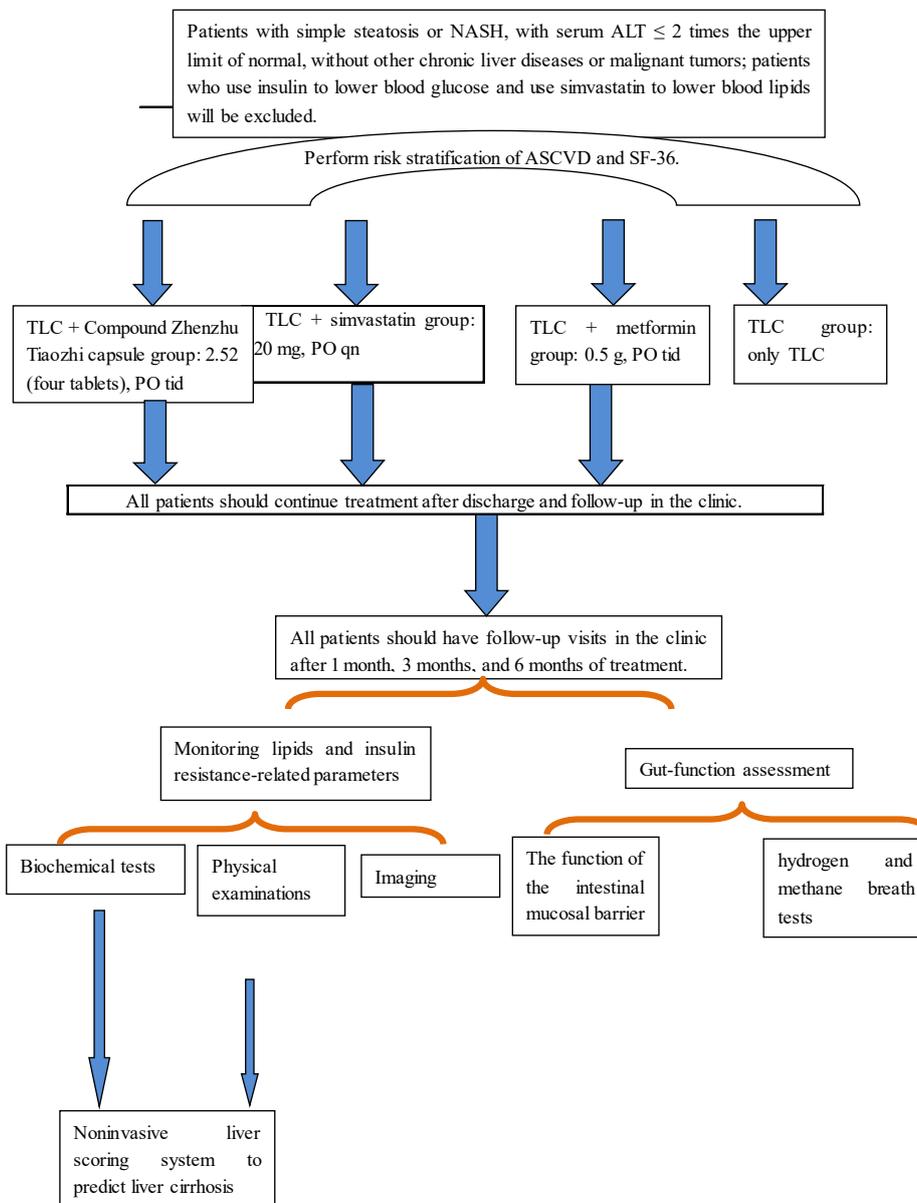


Group 1: treatment with Compound Zhenzhu Tiaozhi capsules

Group 2: treatment with simvastatin

Group 3: treatment with metformin

Group 4: TLC (transform life custom)



ALT, alanine transaminase; ASCVD, atherosclerotic cardiovascular disease

Biochemical tests include routine blood test, liver function tests including measurement of transpeptidases, uric acid, FBS (fasting blood glucose), FINS (fasting insulin), and lipid profiles.

Physical examinations include waist circumference, hip circumference, blood pressure, heart rate, and body mass index (BMI).

Imaging include abdominal color doppler ultrasound, and Fibrotouch

3.2 The Method of Random Assignment

Newly diagnosed patients who meet the inclusion criteria will have a case number according to their order of enrollment (for example: case number of the first patient: 001, second patient: 002). They

will then obtain the corresponding therapeutic regimens based on their case number with 1:1:1:1 ratio randomized tables.

3.3 Random Numbers

Researchers will screen subjects based on the established inclusion and exclusion criteria first, and then assign the case number to the enrolled subjects based on their order of enrollment. Secondly, the enrolled subjects will obtain their corresponding therapeutic regimens based on the assigned case number. Thirdly, researchers will record the case number and therapeutic regimens for each subject in each case report form. The case number for each subject will not change during the entire study.

3.4 Sample Size

A total of 196 patients will be enrolled in this study. The sample size is estimated as follows:

Previous studies have reported that the efficiency rate of metformin and simvastatin in the treatment of NAFLD is 36-52%. We hypothesize that the efficiency rate of Compound Zhenshu Tiaozhi capsules will increase by 25% compared to standard drugs.

Statistical calculation: $P_1 = 69\%$, $P_2 = P_3 = 44\%$, $P_4 = 10\%$, $\delta = P_1 - P_2 = 25\%$, with $\alpha = 0.05$,

power = 0.80, and a bilateral test, $n = \frac{\chi_{\alpha}^2 + \chi_{\beta}^2}{w^2}$, of which $w = \sqrt{\sum_{i=1}^m \frac{(P_{t,i} - P_{c,i})^2}{P_{c,i}}}$, thus n will be equal to 44.

Assuming that the rate of loss to follow up is 10%, each group will require 49 cases. Therefore, a total of 196 cases will be required for the four groups.

4. Evaluation of the Efficacy of Parameters and Predictors

4.1 The Primary Efficacy Parameters

Fat attenuation index: The fat attenuation index will be used to assess the therapeutic efficacy. A normal fat attenuation index is defined as < 240 db/m, mild is 240-264 db/m, moderate is 265-294 db/m, and severity is > 295 db/m. We will detect the fat attenuation index at 0, 1, 3, 6 months following treatment. Effective: fat attenuation index is reduced by a level or more (example: moderate to mild). Invalid: fails to meet the effective standard.

4.2 The Secondary Efficacy Parameters

(1) *Serum triglyceride*

(2) *Serum cholesterol*

(3) *Serum lipoproteins*: chylomicron (CM), very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

(4) *Hydrogen/methane breath testing*: breath testing for SIBO.

(5) *Intestinal mucosal barrier*: detect levels of diamine oxidase (DAO), D-lactic acid (D-lac), and lipopolysaccharide (LPS), and combine with the clinical manifestations to evaluate the function of the intestinal mucosal barrier.

(6) *Quality of life (SF-36 Scores)*: The quality of life of the patients before and after treatment will be assessed using SF-36 scores and compared to assess the improvement.

4.3 Safety

Safety profiling includes adverse events and serious adverse events, as well as vital signs and laboratory tests.

4.4 Predictors

Univariate and multivariate statistical analyses will be used to analyze therapeutic efficiency.

1. Factors considered for univariate analysis are as follows:

- a. Baseline: life style, gender, age, baseline liver biochemical parameters, baseline fat attenuation index, baseline lipid profiles, baseline quality of life, baseline BMI;
- b. Factors relevant to treatment: dose of medication, insulin resistance, intestinal barrier function, SIBO.

2. Factors considered in multivariate analysis are as follows:

Only factors that have statistical significance in univariate analysis will be entered for multivariate analysis. Additional three factors including insulin resistance, intestinal barrier function, and intestinal bacterial overgrowth will still be considered for multivariate analysis, regardless of univariate analysis results.

5. Definitions

Diabetes: fasting blood glucose ≥ 7.8 mmol/L, or two hours post-75 g glucose drink/or postprandial random glucose ≥ 11.1 mmol/L.

Overweight: BMI > 28 (kg/m²); obese: BMI > 31 (kg/m²); BMI = weight/height² (kg/m²)

Liver biochemical parameters: Detect liver injury and liver excretion function at time of admission and 6 months later.

NAFLD Fibrosis score (NFS): This is a noninvasive method to evaluate liver fibrosis, and is calculated by six variables such as age, BMI, and platelet count. The formula is as follows: $-1.675 + (0.037 \times \text{age}) + (0.094 \times \text{BMI}) + (1.13 \times (\text{hyperglycemia or diabetes; yes} = 1, \text{no} = 0)) + (0.99 \times \text{AST} / \text{ALT}) - (0.013 \times \text{platelet count (x } 10^9 / \text{L)}) - (0.66 \times \text{albumin (g / dL)})$. A score less than -1.455 is used to rule out advanced liver fibrosis.

SIBO: Due to small intestinal stasis, bacteria overgrow in the small intestine, resulting in several clinical symptoms such as malabsorption, diarrhea, anemia, and nutrient absorption disorders. Appropriate use of antibiotic can achieve beneficial effects.

Intestinal barrier function test: detect DAO, D-lac, and LPS, and combine with clinical manifestations to evaluate the function of intestinal mucosal barrier.

SF-36 assessment: SF-36 can comprehensively assess the quality of life from multiple dimensions, and is now widely applied in the evaluation of quality of life, clinical trial results, and clinical efficacy.

6. Definitions of Data Sets

6.1 Full Analysis Set (FAS)

The FAS refers to the combination of qualified cases and shedding cases, but without exclusion cases. Baseline and demographic characteristics will be analyzed. Intention to treat (ITT) analysis will be used in the absence of primary outcome measures.

6.2 Per Protocol Set (PPS)

The PPS refers to the set of subjects who meet the inclusion criteria and complete the research project, which also means they comply with the research protocol, have good compliance, do not use banned substances during trial, and have completed the case report form (CRF). The PPS analysis is mainly used to assess primary efficacy.

6.3 Safety Set (SS)

SS analysis will be performed for all randomized subjects who received at least one dose of the study drugs and have at least one safety assessment. The missing value of safety cannot be carried forward. Some excluded cases can be evaluated, such as a case whose age exceeds the inclusion criteria, but will not include cases who use prohibited drugs. The rate of adverse events will be calculated using the total cases in the SS as the denominator.

7. Interim Analysis

Some specialized staff in the Department of Statistics will be responsible for the real-time statistical analysis of the study data and will present the results with an analysis report.

8. Data Verification

The final data analysis should not be started until all data have been verified, questions have been resolved, and the databases have been locked. We aim to demonstrate that the source of the data is real and valid, and will ensure that the database is locked prior to data analysis.

8.1 Data Processing and Transmission

All data will be recorded in a SAS® data set format using Data Management Center (SAS 9.3 or higher version).

8.2 Record Lock and Data Export

When subjects complete the trial, their CRFs will be subjected to auditors to verify the data. Once the data from each subject is verified, the data managers will lock all data files. After all data is locked, data managers will be responsible for importing the data sets into the designated database and transferring the data sets to statistical staff members for statistical analysis.

9. Statistical analysis

9.1 Case distribution

Descriptive statistical analysis will be performed for all enrolled cases, randomized cases, completed cases, and uncompleted cases, classified according to whether they meet the inclusion and exclusion criteria, whether they have signed the informed consent form, and other classifications. The data from enrolled subjects will be also used to conduct descriptive statistical analysis according to the definitions of FAS, PPS and SS. The frequency and constituent ratio will be used when appropriate.

9.2 Important Protocol Deviations

Important Protocol Deviations (IPD) will be described using cases and composition ratios. The IPD for each enrolled subject will be listed.

9.3 Processing of Missing Values

Results will be carried forward when primary outcome measures are absent, according to the following details:

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1. If patients lack a fat attenuation value at the 6 months follow up and they do not show a response at the cessation of therapy, the results at cessation of therapy can be carried forward to the 6 months follow up.
 2. If patients withdraw from the study before cessation of the study, and continue without a response, the results of the last visit can be carried forward.
 3. If patients lack an intermediate visit of a fat attenuation value, but have results for insulin resistance, intestinal barrier function, and intestinal bacterial overgrowth, the previous results can be carried forward.
 4. If patients withdraw from the study because of a full recovery, the results of the last visit can be carried forward.

9.4 Combination Medications

All drugs will be classified using the Anatomical Therapeutic Chemical (ATC) classification system based on the World Health Organization (WHO) Drug Dictionary, and a detailed list will be made to calculate the cases and the composition ratios of combination medications.

9.5 Baseline characteristics

According to the definition of SS, the demographic and baseline characteristics, as well as vital signs will be described. Qualitative data analysis includes the number of cases and composition ratios. Quantitative data analysis includes the number of cases, mean, standard deviation, median, maximum and minimum values, upper and lower quartiles (Q1 and Q3), and confidence interval. Chi-square test, rank-sum test, and univariate analysis will be used when appropriate.

Screening and baseline indicators are as follows:

Date of birth, gender, race, marital status, occupation, pregnancy test results, medical history, pathways of hepatitis infection, time of infection, and laboratory tests.

- History: changes to or any new combinations of medications
- Physical examinations

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- Vital signs: blood pressure, pulse, heart rate, respiration, body temperature, height, weight, BMI
 - The physical examinations including skin, head, eyes, ears, nose and sinuses, mouth and pharynx, neck, back, breasts and armpits, chests and lungs, heart, abdomen, rectum and anus, reproductive organs, legs, musculoskeletal system, and blood vessels.
 - Urine indicators
 - pH, protein, glucose, acetone body, white blood cell (WBC) and red blood cell (RBC) counts
 - Hematology indicators
 - Routine blood test: hemoglobin, RBC, WBC, absolute neutrophil count, absolute lymphocyte count and classification, platelet count
 - Prothrombin time
 - Biochemistry tests: alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (Tbil), direct bilirubin (Dbil), total protein (TP), albumin (ALB), Gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), creatinine (Cr), glucose (Glu), triglyceride (TG), cholesterol (Cho), blood urea nitrogen (BUN) and (low density lipoprotein) LDL
 - Viral hepatitis biomarkers
 - Hepatitis B surface antigen and antibody (HBsAg, anti-HBs)
 - Hepatitis B e antigen and antibody (HBeAg, HBeAb)
 - Hepatitis B core antibody (HBcAb)
 - Human immunodeficiency virus (HIV)-Ab, HIV RNA
 - Hepatitis B virus (HBV) DNA
 - Hepatitis C virus (HCV) RNA
 - Serum alpha-fetoprotein (AFP) detection
 - Chest X-ray
 - Electrocardiogram (ECG)
 - Abdominal ultrasound
 - Left hepatic lobe (cm), right hepatic lobe (cm), sphincter diameter (cm), portal vein diameter (cm), spleen thickness (cm), spleen length (cm), and splenic vein diameter (cm)

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- Quality of life
 - SIBO
 - Insulin resistance levels