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Analysis of traditional Chinese medicine syndrome types and frequency changes of CD^{8+} and CD^{25+} T cells in metabolic-related fatty liver disease

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ABSTRACT

This study aimed to observe and analyze the changes in traditional Chinese medicine (TCM) syndrome types and CD^{8+} and CD^{25+}T cell frequencies of metabolic-related fatty liver disease. For this purpose, 100 patients with metabolic-related fatty liver disease and their TCM syndrome types who were screened for medical treatment were collected. Flow cytometry was used to detect the changes in the frequency of CD^{8+} and CD^{25+}T cells in the peripheral blood of patients, as well as liver function, fasting blood glucose, and lipid index. The frequency differences of CD^{8+} and CD^{25+}T cells in patients with different syndrome types were compared. To use partial correlation analysis to determine the correlation between CD^{8+}, CD^{25+}T cell frequencies and TCM syndrome types in patients. Results showed that a total of 30 cases of liver stagnation and spleen deficiency syndrome, 25 cases of phlegm turbidity internal obstruction syndrome, 20 cases of dampness heat stasis syndrome, and 25 cases of phlegm stasis mutual accumulation syndrome were included in the 100 MAFLD patients. There was statistical significance ($P<0.01$) in the comparison of ALT, AST, GGT, TC, TG, HDL-C, LDL-C, FPG, HOMA-IR, $CD^{8+}CD^{25+}$, $CD^{8+}CD^{25-}$, $CD^{8-}CD^{25+}$, and $CD^{8-}CD^{25-}$ among patients with different TCM syndrome types. There is a positive correlation between TCM syndrome types and patients' $CD^{8+}CD^{25+}$, $CD^{8+}CD^{25-}$, and $CD^{8-}CD^{25+}$, while there is a negative correlation between them ($P<0.05$). From the chord diagram, the relationship between $CD^{8+}CD^{25+}$ and TCM syndrome types is the closest. The ROC curve was used to analyze and determine that the relevant standard for $CD^{8+}CD^{25+}$ in liver depression and spleen deficiency syndrome is <4.90%; The relevant standard for phlegm turbidity internal obstruction syndrome is 4.90%~7.88%; Damp heat stasis syndrome is 7.88%~8.20%; The syndrome of phlegm and blood stasis accumulation is more than 8.20%. The TCM syndrome types of metabolic-related fatty liver disease will vary with the frequency of CD^{8+} and CD^{25+}T cells. In conclusion, TCM syndrome types are closely related to the severity of the patient's condition and immune function, providing a new perspective and means for understanding the pathogenesis of metabolic-related fatty liver disease and evaluating the condition.

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Introduction

Metabolically Associated Fatty Liver Disease (MAFLD) is the most common chronic liver disease worldwide, which can develop into cirrhosis, hepatocellular carcinoma, and liver failure (1). According to the "Report on the Nutrition and Chronic Disease Status of Chinese Residents", the prevalence of obesity in China further increased in 2019 compared to 2012, with an overall obesity population exceeding 250 million, and there is still a continuous upward trend (2). Research suggests that the prevalence of MAFLD in morbidly obese individuals is 65-93% (3). MAFLD has become a high incidence and common disease in China. The occurrence of MAFLD is related to various factors such as obesity, insulin resistance, and abnormal lipid metabolism. In 2020, numerous scholars jointly demonstrated the inaccuracy of naming Non-alcoholic Fatty Liver Disease (NAFLD), renamed it MAFLD, and provided a more detailed disease diagnosis and risk stratification for the first time (4). MAFLD often uses ultrasound for screening and lacks simple and feasible clinical diagnostic criteria. At the same time, MAFLD often merges with other chronic liver diseases, which challenges the exclusive diagnostic strategy of MAFLD. Chinese medicine regards "Gan Dan (liver disease)" as the traditional Chinese medicine (TCM) name for MAFLD, and believes that its etiology and pathogenesis are improper diet, excessive comfort, emotional disorders, phlegm dampness constitution, and age and physical decline. TCM syndrome types (TCMST) can be divided into four types: liver stagnation and spleen deficiency, phlegm turbidity internal obstruction, dampness heat stasis accumulation, and phlegm stasis mutual accumulation. Research shows that the liver microenvironment of chronic steatosis induces the regulatory mechanism of activated CD^{8+}T cells, leading to the presence of liver parenchymal and non-liver parenchymal cells in patients (5). The pathogenesis of MAFLD involves many immune cell-mediated inflammatory processes, and the relationship between the frequency changes of CD^{8+} and CD^{25+}T cells and the TCMST of patients still needs further verification. This study aims to observe and analyze the changes in TCMST and CD^{8+}, CD^{25+}T cell frequency of MAFLD.

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Materials and Methods

General materials

100 MAFLD patients who were hospitalized from Jan 2020 to Dec 2022. Patients included in the inclusion criteria: (I) Liver ultrasound examination meets the diagnostic criteria for diffuse fatty liver; (II) Between the ages of 18 and 70, regardless of gender; (III) Agree to participate in this study and sign an informed consent form, and be able to complete a questionnaire survey, participate in four diagnostic tests, and undergo biochemical tests; (IV) With metabolic syndromes such as abnormal glucose tolerance, hyperuricemia, and fat metabolism disorder. Exclusion criteria: (I) Patients with alcoholic fatty liver or viral hepatitis-related fatty liver; (II) Those with severe heart, lung, kidney diseases or malignant tumors; (III) Those who have taken immunomodulatory drugs within the past month; (IV) Patients who are unable to obtain complete case data; (V) Pregnant and lactating women; (VI) HIV infected individuals or AIDS patients with CD^{4+}T cell absolute value<200cells/μL; (VII) Patients with other liver diseases such as viral hepatitis, autoimmune liver disease, hepatolenticular degeneration, liver cancer, or serious diseases such as respiratory system, blood system, urinary system, endocrine system, etc.

General situation investigation

The study used a questionnaire survey method to collect patients' age, gender, course of disease, and clinical symptoms. A self-made general situation questionnaire was used, and the questionnaire survey was completed by two professional scale investigators.

Observation target

TCMST

Patients were classified based on their clinical manifestations and pulse patterns by TCM experts with senior professional titles. According to the consensus opinion on the diagnosis and treatment of NAFLD integrated traditional Chinese and Western medicine (6), the symptoms were divided into four types: liver depression and spleen deficiency syndrome (LDSDS), phlegm turbidity internal obstruction syndrome (PTIOS), dampness heat stasis syndrome (DHSS), and phlegm stasis mutual accumulation syndrome (PSMAS). Having 2 corresponding main syndromes and 1 or 2 secondary syndromes, and evaluating the syndrome type based on the results of tongue pulse and physical and chemical examinations. Please refer to Table 1 for the standards of tongue and pulse for the main and secondary syndromes.

Examination of T cell frequency and related indicators

5ml of fasting venous blood of the subjects was collected in the morning of the next day after 12-hour fasting and added to the EDTA anticoagulant tube. Single nuclear cells were isolated by density gradient centrifugation (Ficoll separation solution with a specific gravity of 1.077g/ml) after diluting the extracted whole blood. The centrifugation conditions were 2500r/min at room temperature and 20min. The supernatant was discarded, PBS (including 2% FBS) was added for resuspension and washing twice, at 2400r/min, and centrifuged for 10 minutes. The supernatant was discarded, and the cells were suspended in 300μL of PBS. 1 ml of collagen IV 0.1mg/ml and DNase I 20μg/ml were added. It was digested in a 37°C water bath for 20min, centrifuged, and the supernatant was discarded. It was washed twice with PBS. A 10μl cell suspension was prepared, counted microscopically, and the cell density was adjusted to 106×1/ml. A 100μl of cell suspension (1×106 cells) was taken and dual color labeled antibodies such as CD8-PE and CD25-FITC were added. The mixture was then incubated at room temperature in the dark for 30 minutes. It was washed twice with PBS, centrifuged, and the supernatant was discarded with 300 microliters of PBS resuspension. It was then detected in the machine (flow cytometry FACS Calibur). CellQuest and FlowJo software were used for data collection and analysis to obtain the frequency (%) of CD^{8+} and CD^{25+}T cells in CD^{4+}/CD^{8+}T cells. Meanwhile, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-Gamma-glutamyl transferase (GGT) and total cholesterol (TC) were detec-

Table 1. TCM main syndrome evaluation scheme for MAFLD.

TCMST	Main syndromes	Secondary syndromes	Tongue pulse
LDSDS	① Thoracic rib fullness, ② Depression and discomfort ③ Fatigue ④ Abdominal pain and diarrhea	① Abdominal distension and discomfort ② Loss of appetite ③ Nausea and vomiting ④ Irregular bowel movements ⑤ Loss of appetite	① Tongue light red ② Thin white or white coating ③ Tooth marks ④ Fine pulse string
PTIOS	① Obesity in body posture ② Discomfort or tightness in the right flank ③ Overall fatigue and heaviness ④ Sticky and uncomfortable stools	① Wrist and abdominal distension ② Fatigue and weakness ③ Loss of appetite ④ Dizziness and nausea	The tongue is light in texture, with a white and greasy coating and smooth veins.
DHSS	① Pain and swelling in the right rib region; ② Trapped and heavy all over; ③ Abdominal distension or pain; ④ Stool is sticky and unpleasant.	① Yellowing of body and eyes; ② Yellow urine color; ③ Stickiness in the mouth; ④ Dry mouth and bitter mouth	The tongue is red in texture, the tongue coating is yellow and greasy, and the veins are smooth or moist.
PSMAS	① Thoracic or dull pain in the ribs; ② Subclavian lumps; ③ Dark complexion; ④ Body obesity.	① Chest courtyard fullness; ② Spitting and salivating; ③ Nadazhao oil; ④ Heavy limbs	The tongue is dark red with ecchymosis, and the body is plump with teeth marks on the edges. The coating is greasy, and the veins are smooth or astringent.

ted using enzymatic methods; Triglycerides (TG) were detected using the GPO-PAP colorimetric method; High-density lipoprotein cholesterol (HDL-C) was detected by direct method; Low-density lipoprotein cholesterol (LDL-C) is calculated using the Friedewald formula: LDL-C=TC - HDL-C -TG/5 (mmol/L); Fasting plasma glucose (FPG) was measured using the GOD-PAP colorimetric method; The product used a simplified absorbance method to detect absorbance at a wavelength of 510nm and calculate concentration. The above experimental data was collected and radioimmunoassay was used to measure serum FINS. By using serum FPG and FINS to obtain insulin resistance index, the specific formula was: HOMA-IR=FPG (mmol/L)×FINS (mU/L)/22.5.

Statistics

The software SPSS 26.0 is used to measure the data and the data conforming to normal distribution is represented by $\bar{x}\pm s$; The measurement data of non-normal distribution is expressed by M (interquartile distance), and the data between different syndrome types are analyzed by multi-group variance analysis. When the results have statistical significance, further LSD-t test is performed for pairwise comparison; the Kruskal Wallis H test is performed when the data does not conform to normal distribution. Utilization of counting data between different syndrome types using Table R×C to calculate and select χ^2 test based on the expected value T frequency. Partial correlation analysis can determine the correlation of various indicators, and R software can be used to draw a correlation chord diagram. To calculate the maximum indicator of correlation using Medcalc software for TCMST-related standards.

Results

Patient's TCMST distribution

There were a total of 30 cases of LDSDS, 25 cases of PTIOS, 20 cases of DHSS, and 25 cases of PSMAS in 100 MAFLD patients. There was no statistically significant difference in gender distribution among different syndrome types (P>0.05); There is statistical meaning (P<0.01) in the comparison of age and disease course among different syndrome types, as shown in Table 2.

Distribution of metabolic-related indicators in different TCMSTs

The comparison of ALT, AST, GGT, TC, TG, HDL-C, LDL-C, FPG, and HOMA-IR among different TCMST patients is demonstrated in Table 3 (P<0.01).

Frequency distribution of $CD^{8+}$and CD^{25+}T cells in patients

Table 4 shows the comparison of $CD_8^+CD_{25}^+$, $CD_8^+CD_{25}^-$, $CD_8^-CD_{25}^+$, $CD_8^-CD_{25}^-$ among different TCMST patients (P<0.01).

Partial correlation analysis of TCMST with CD^{8+} and CD^{25+}T cells in patients

Assign LDSDS to 1, PTIOS to 2, DHSS to 3, and PSMAS to 4; Take TCMST, ALT, AST, GGT, TC, TG, HDL-

Table 2. TCMST and general data analysis of MAFLD.

TCMST	Case	Gender		Age (year)	Course of disease (year)
		Male	Female		
LDSDS	30	18	12	45.87±14.88^{d}	7.33±3.28^{cd}
PTIOS	25	13	12	44.76±10.45^{d}	5.60±3.01^{cd}
DHSS	20	12	8	40.15±10.96^{d}	2.85±1.39^{ad}
PSMAS	25	14	11	53.72±13.63^{abc}	12.24±4.31^{abc}
F/χ^2		0.449		4.456	34.092
P		0.930		0.006	0.000

Note: Compared with LDSDS, ^{a}P<0.05; Compared with PTIOS, ^{b}P<0.05; Compared with DHSS, ^{c}P<0.05; Compared with PSMAS, ^{d}P<0.05.

Table 3. Distribution of metabolic-related indicators of different TCMSTs in MAFLD.

TCMST	n	ALT (U/L)	AST (U/L)	GGT (U/L)	TC (mmol/L)	
LDSDS	30	34.92±14.05^{bcd}	35.81±16.04^{bd}	40.12±20.23^{d}	5.13±1.08^{d}	
PTIOS	25	60.23±27.69^{a}	49.30±17.86^{ad}	53.55±23.03^{d}	4.83±1.40^{cd}	
DHSS	20	74.54±29.08^{a}	51.38±23.63^{ad}	53.37±29.62^{d}	5.98±1.83^{c}	
PSMAS	25	72.80±30.17^{a}	72.13±28.22^{abc}	73.36±28.19^{abc}	6.70±2.25^{ab}	
F		13.939	12.954	8.033	6.536	
P		0.000	0.000	0.000	0.000	
TCMST	**n**	**TG (mmol/L)**	**HDL-C (mmol/L)**	**LDL-C (mmol/L)**	**FPG (mmol/L)**	**HOMA-IR**
LDSDS	30	1.84±0.67^{cd}	1.10±0.31^{cd}	3.08±0.86^{cd}	5.21±0.37^{cd}	2.32±0.99^{bcd}
PTIOS	25	2.04±0.98^{cd}	0.98±0.20^{d}	3.58±0.89^{d}	5.86±1.19^{acd}	3.57±1.64^{ad}
DHSS	20	2.86±1.53^{abd}	0.88±0.19^{a}	4.09±1.36^{ad}	7.19±1.67^{ab}	4.78±2.75^{a}
PSMAS	25	4.05±1.61^{abc}	0.76±0.20^{ab}	4.91±1.61^{abc}	6.35±1.72^{ab}	5.83±3.02^{ab}
F		17.744	10.053	11.305	14.815	12.966
P		0.000	0.000	0.000	0.000	0.000

Note: Compared with different syndromes: LDSDS, ^{a}P<0.05; PTIOS, ^{b}P<0.05; DHSS, ^{c}P<0.05; PSMAS, ^{d}P<0.05.

Table 4. Distribution of $CD^{8+}$and CD^{25+}T cell frequencies in MAFLD (x ± s, %).

TCMST	n	$CD_8^+CD_{25}^+$	$CD_8^+CD_{25}^-$	$CD_8^-CD_{25}^+$	$CD_8^-CD_{25}^-$
LDSDS	30	$3.14±1.14^{bcd}$	$30.30±6.45^{cd}$	$7.03±2.23^{bcd}$	$62.00±9.45^{bcd}$
PTIOS	25	$5.22±2.07^{acd}$	$33.81±8.95^{d}$	$9.63±2.68^{acd}$	$51.31±9.38^{acd}$
DHSS	20	$8.20±3.36^{abd}$	$36.19±12.62^{ad}$	$13.26±3.87^{abd}$	$38.91±8.11^{abd}$
PSMAS	25	$11.26±4.54^{abc}$	$42.75±10.02^{abc}$	$16.47±7.05^{abc}$	$32.76±8.95^{abc}$
F		37.682	8.242	24.292	55.215
P		0.000	0.000	0.000	0.000

Table 5. Partial correlation analysis of TCMST with CD^{8+} and CD^{25+}T cells.

Variables	TCMST	$CD_8^+CD_{25}^+$	$CD_8^+CD_{25}^-$	$CD_8^-CD_{25}^+$	$CD_8^-CD_{25}^-$
TCMST	1.000				
$CD_8^+CD_{25}^+$	0.715^{**}	1.000			
$CD_8^+CD_{25}^-$	0.408^{**}	0.350^{**}	1.000		
$CD_8^-CD_{25}^+$	0.631^{**}	0.400^{**}	0.301^{**}	1.000	
$CD_8^-CD_{25}^-$	-0.795^{**}	-0.525^{**}	-0.437^{**}	-0.614	1.000

Note: * * P<0.05.

C, LDL-C, FPG, HOMA-IR, $CD_8^+CD_{25}^+$, $CD_8^+CD_{25}^-$, $CD_8^-CD_{25}^+$ and $CD_8^-CD_{25}^-$ as variables; Calculate the correlation of each variable using age and disease course as control variables. There is a positive correlation between TCMST and patients' $CD_8^+CD_{25}^+$, $CD_8^+CD_{25}^-$, and $CD_8^-CD_{25}^+$, while there is a negative correlation between TCMST and $CD_8^-CD_{25}^-$ (P<0.05) (Table 5); From the chord diagram (Figure 1), the relationship between $CD^{8+}CD^{25+}$ and TCMST is the closest. The ROC curve analysis determined that the relevant standard for $CD^{8+}CD^{25+}$ in LDSDS was<4.90%; The relevant standards for PTIOS range from 4.90% to 7.88%; DHSS is 7.88%~8.20%; PSMAS is >8.20%.

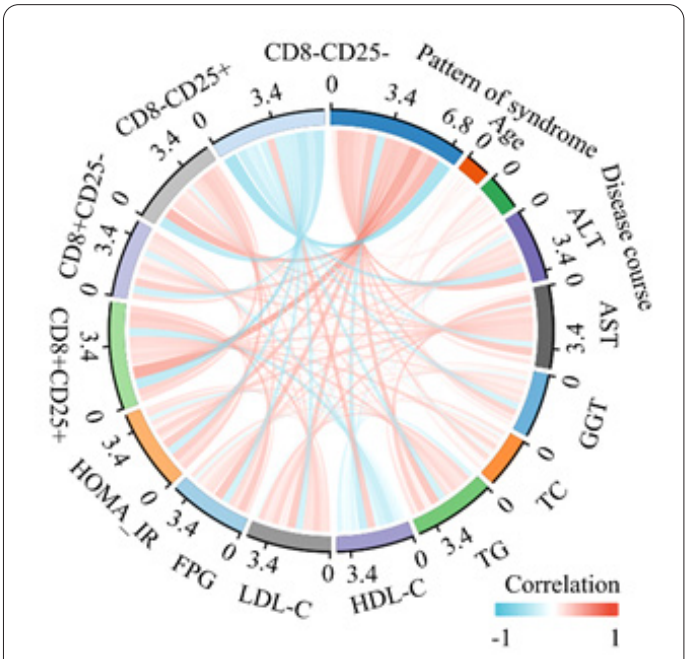

Figure 1. Correlation and chord chart of various indicators.

Discussion

The liver is the central metabolic organ in the body that regulates energy and lipid metabolism and has powerful immunological functions. Obesity and sedentary lifestyles lead to excess liver metabolic capacity, leading to liver lipid accumulation, chronic necrotizing inflammation, enhanced mitochondrial/ER stress, and the occurrence of NAFLD. In recent years, many potential multifactorial causes of MAFLD have been identified, and the cellular mechanisms that maintain disease development have been isolated to the single-cell level (7). MAFLD patients will suffer from insulin resistance, intestinal ecological imbalance, and even prefrontal cortex abnormalities under the influence of a high inflammatory state related to persistent liver steatosis (8). MAFLD progression to hepatitis is closely related to a variety of metabolic diseases including insulin resistance, obesity, diabetes and metabolic syndrome (9). Fatty liver mice that did not experience dietary induction mainly played a role in the initiation and progression of liver inflammation (10). It can be seen that stress, inflammation, insulin resistance and other issues are the main reasons for the progression of MAFLD.

LDSDS is the most common TCMST in MAFLD, and patients are often in the early stages of the disease, accompanied by insulin resistance, elevated blood sugar, elevated cholesterol and triglyceride levels. PTIOS is usually accompanied by elevated levels of cholesterol and triacylglycerol, mainly due to lipid metabolism disorders and blocked cholesterol excretion. DHSS has abnormalities in fat metabolism, carbohydrate metabolism, liver function, and is accompanied by chronic inflammation and water electrolyte disorders. PSMAS is more severe than DHSS. The four types of TCMST change with the aggravation of the disease. This study observed a total of 100 MAFLD patients, including 30 LDSDS, 25 PTIOS, 20 DHSS, and 25 PSMAS. The distribution of the four TCMSTs is relatively balanced. This result is similar to the research of scholars (11,12). Previous diagnosis of diseases often used liver function, blood glucose, and lipid indicators for clinical differentiation. However, due to the need for identification and observation of multiple indicators, it is difficult to meet clinical needs, and it is still necessary to explore more convenient and efficient evaluation indicators.

The results of this study showed that there was statistical significance (P<0.01) in the comparison of ALT, AST, GGT, TC, TG, HDL-C, LDL-C, FPG, HOMA-IR, $CD_8^+CD_{25}^+$, $CD_8^+CD_{25}^-$, $CD_8^-CD_{25}^+$, $CD_8^-CD_{25}^-$ among different TCMST patients; There are significant distinctions in liver function, blood lipid and blood glucose indicators,

CD_8, and CD_zT cells among patients with different syndrome types. At the same time, there is a positive correlation between TCMST and patients' $CD_8^+CD_{25}^+$, $CD_8^+CD_{25}^-$, and $CD_8^-CD_{25}^+$, while there is a negative correlation between TCMST and $CD_8^-CD_{25}^-$ ($P<0.05$). The occurrence of MAFLD involves the immune system. Scholars have analyzed the activation profiles of iNKT cells, natural killer gene 2 members, and CD_4/CD_8T cells in peripheral blood mononuclear cells of NAFLD patients and DILI patients with or without significant liver fibrosis. CD_8T cells were identified as a potential biomarker for distinguishing between liver fibrosis and MAFLD (13). In addition, animal experiments have found that CD_8T lymphocytes exhibit a decrease in CD_{25} activation markers under the action of co-stimulatory molecules expressed in dendritic cells (14). $CD^{8+}CD^{25+}$ plays an important role in the immune cell function during the occurrence and development of MAFLD.

Further combined with the chord diagram, it was found that the $CD^{8+}CD^{25+}$ is the most closely related to TCMST. Research has shown that CD^{8-} positive T-lymphocytes dominate in the study cases. There was a significant correlation ($P<0.01$) between the determined NASH and hs-CRP, serum adiponectin, and leptin/adiponectin ratio (15). Moreover, CD^8 is closely related to liver fibrosis (16). The percentage of pro-inflammatory macrophages, pro-inflammatory CD^4 and CD^8 T cell populations, and the area under the plasma PAI-1 concentration curve are negatively correlated with liver and systemic insulin sensitivity (17). NAFLD and liver inflammation in hepatocellular carcinoma patients, as well as upregulation of CD^8 in situ, are key factors/determinants of liver disease progression (18). Compared with CD^{4+}, CD^{8+} has a stronger effect on the proliferation of T cells ($CD^{4+}CD^{25-}$) and the generation of Th1 cytokines, and the inhibitory ability of $CD^{8+}CD^{25+}$ is stronger. So far, most studies have been limited to the immunosuppressive effects of $CD^{8+}CD^{25+}$ in malignant and autoimmune diseases. Some studies also found that the proportion of $CD^{8+}CD^{25+}$ decreased in diabetes patients, and the insulin resistance index increased (19). The changes in CD^{4+} and CD^{8+}T lymphocytes in the peripheral blood of NAFLD patients are opposite to those in the liver, with a significant decrease in CD^{8+}T cells, which continue to worsen with the severity of fatty liver disease (20). The pathogenesis of diabetes and MAFLD is similar; both of them will produce a micro-inflammatory reaction. The reduction of $CD^{8+}CD^{25+}$ can lead to an increase in the secretion of inflammatory cytokines, further exacerbating the severity of the disease. Research has found that knocking out CD^8T lymphocytes in mice leads to a decrease in inflammation levels and liver fibrosis levels. CD^{8+}T lymphocytes can promote natural killer cell T-lymphocytes and promote the occurrence of hepatocellular carcinoma (21). This study further utilized ROC curve analysis to determine that the relevant standard for $CD^{8+}CD^{25+}$ in LDSDS is <4.90%; The relevant standards for PTIOS range from 4.90% to 7.88%; DHSS is 7.88%~8.20%; PSMAS is >8.20%. However, due to the small sample size collected by our research institute, further large-scale studies are needed to verify the accuracy of our research results.

In summary, the TCMST of MAFLD varies with the frequency of CD^{8+} and CD^{25+}T cells. TCMST is closely related to the severity of the patient's condition and immune function, providing a new perspective and means for understanding the pathogenesis of MAFLD and evaluating the condition.

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