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Original Article

Correlation of CD3+/CD4+, and serum CK-18 fragment levels with glucose and lipid metabolism in elderly type 2 diabetes patients with nonalcoholic fatty liver disease

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Abstract: Objective: To test the correlation of helper T lymphocytes (CD3+/CD4+), and cytokeratin 18 fragment (CK-18) with glucose and lipid metabolism in elderly patients with type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD). Methods: A total of 108 patients with T2DM hospitalized in Geriatrics, Taizhou People's Hospital from August 2019 to December 2020 were obtained and grouped into' Non-NAFLD group (58 patients) and NAFLD group (50 patients) according to the patients' conditions. Another 50 healthy people were obtained as the control group (CG). The BMI was tested, and the elbow venous blood was collected. The indexes of blood glucose, liver and kidney function (ALT, AST, creatinine, urea nitrogen), blood lipid (triglyceride, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol) and blood uric acid were detected. CD3+/CD4+ in elbow venous blood was tested using flow cytometry, and CK-18 was tested using ELISA. Pearson correlation coefficient was applied to test the correlation of CD3+/CD4+, CK-18 with glucose and lipid metabolism in NAFLD group. Results: Compared with the CG, CK-18 in the other two groups were elevated, and CK-18 in the NAFLD group were elevated compared to the Non-NAFLD group. Compared with the CG, CD3+ and CD4+ in the other two groups were decreased, and CD3+ and CD4+ in the NAFLD group decreased compared to the Non-NAFLD group. Correlation analysis revealed that both CD3+ and CD4+ had a negative correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL and LDL, while CK-18 had a positive correlation with these indexes. ROC curve revealed that the AUC values of CK-18, CD3+ and CD4+ for NAFLD in elderly T2DM patients were 0.875, 0.867, and 0.871, respectively. Logistic regression analysis revealed that FINS, HOMA-IR, CK-18, CD3+ and CD4+ were all related factors leading to NAFLD in elderly T2DM patients. Conclusion: CD3+/CD4+, and CK-18 were correlated with glucose and lipid metabolism in elderly T2DM patients with NAFLD. They may be related to the development of T2DM and NAFLD, and these indexes can be used as biological diagnostic indicators for elderly T2DM patients with NAFLD.

Keywords: Type 2 diabetes mellitus, nonalcoholic fatty liver disease, CD3+/CD4+, cytokeratin 18 fragment, glucose and lipid metabolism

Introduction

Diabetes mellitus is a metabolic disorder, which poses a serious threat to human life and health, and its incidence rate is extremely high and is still growing [1]. According to the survey, the global prevalence of diabetes was 9.3% (463 million people) in 2019, and it will rise to 10.2% (578 million people) in 2030 and 10.9% (700 million people) in 2045 [2]. Type 2 diabetes mellitus (T2DM) is the most common in diabetes, and it accounts for more than 90% of the total diabetes cases in China [3]. Non-alcoholic fatty liver disease (NAFLD) is considered the most common chronic liver disease, which is characterized by the accumulation of liver fat more than 5% and has no correlation with excessive intake of alcohol [4]. NAFLD plays a

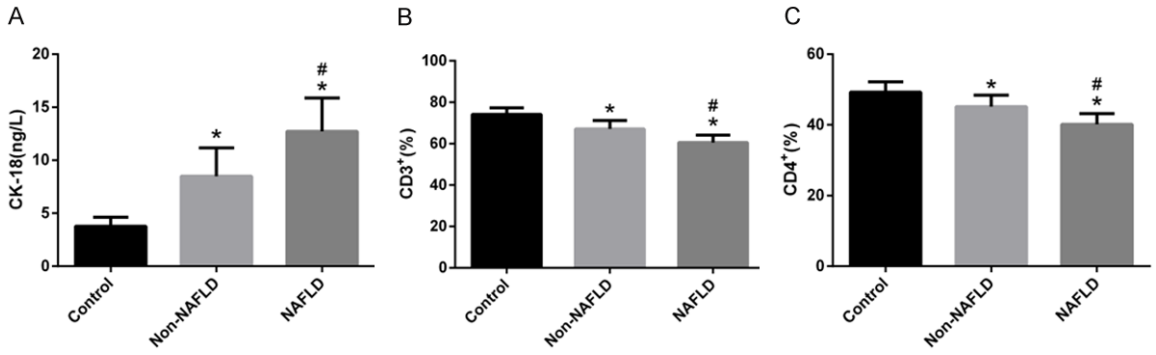

Figure 1. CK-18, CD3+, and CD4+ in the three groups. A: Compared with the control group (CG), CK-18 in the other two groups was elevated, and CK-18 in NAFLD group was elevated compared to the Non-NAFLD group. B, C: Compared with the CG, CD3+, and CD4+ in the other two groups were decreased, and CD3+ and CD4+ in NAFLD group were decreased compared to the Non-NAFLD group ($P < 0.05$). Note: * means compared with the CG, $P < 0.05$; # means compared with Non-NAFLD group, $P < 0.05$.

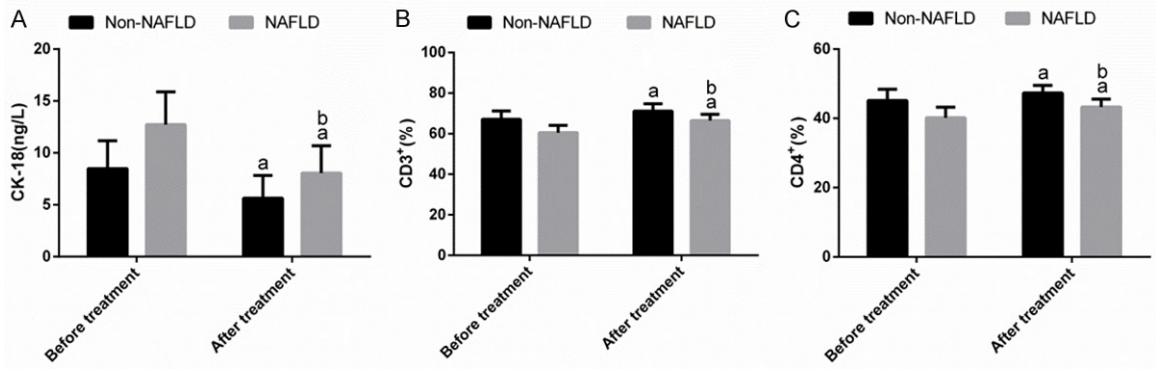

Figure 2. Comparison of CD3+, CD4+, and CK-18 before and after treatment. A: After treatment, CK-18 in both groups was elevated, and CK-18 in NAFLD group was higher than that of the Non-NAFLD group. B, C: After treatment, CD3+ and CD4+ in both groups decreased, and CD3+ and CD4+ in NAFLD group were decreased compared to the Non-NAFLD group. Note: a means a comparison before and after treatment, $P < 0.05$; b means compared to Non-NAFLD group, $P < 0.05$.

Table 1. Comparison of biochemical indexes in the three groups

Group	Control (n=50)	Non-NAFLD group (n=58)	NAFLD group (n=50)
FPG (mmol/L)	5.12±0.72	8.08±1.58*	8.59±1.81*
HbA1C (%)	4.53±0.86	7.82±1.62*	8.62±1.93*
FINS (uU/ml)	4.08±0.49	6.26±1.52*	8.64±1.81*,#
HOMA-IR	1.89±0.63	4.15±1.68*	5.23±2.02*,#
ALT (U/L)	15.15±6.12	32.45±19.26*	58.93±23.35*,#
AST (U/L)	18.26±5.65	28.16±12.09*	30.01±11.48*
TG (mmol/L)	1.12±0.41	2.45±0.68*	3.52±0.86*,#
TC (mmol/L)	4.68±0.59	5.72±0.93*	6.89±1.02*,#
HDL (mmol/L)	1.15±0.26	1.23±0.23	1.26±0.38
LDL (mmol/L)	2.06±0.45	2.52±0.46*	2.63±0.55*
BMI (kg/m^2)	24.16±1.95	25.89±2.35*	26.75±2.48*

Note: *means compared with the controls, $P < 0.05$; #means compared with Non-NAFLD group, $P < 0.05$.

dominant role in chronic liver disease and cirrhosis, and it is a risk factor for hepatocellular carcinoma (HCC) [5]. NAFLD was closely correlated with T2DM, and the two can influence and promote each other. More than three quarters of T2DM patients have NAFLD, which leads to a worse prognosis [6]. Therefore, it is of great clinical significance to further explore the correlation of T2DM with NAFLD.

At present, with the deepening research on T2DM and NAFLD, it was found that these two diseases are closely related to the immune disorders of patients [7, 8]. T lymphocyte subsets are immune-regulated cells.

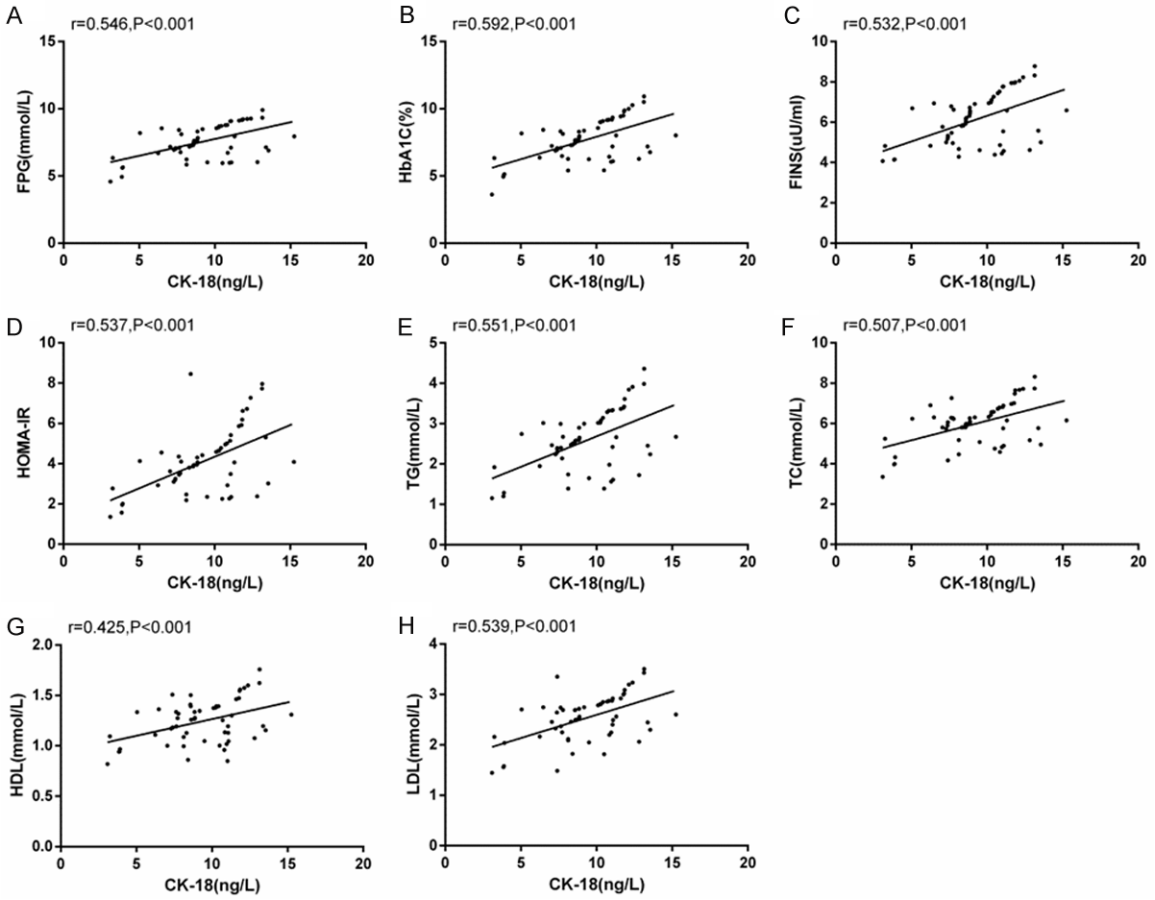

Figure 3. Correlation of CK-18 with glucose and lipid metabolism. A: CK-18 was positively correlated with FPG. B: CK-18 was positively correlated with HbA1C. C: CK-18 was positively correlated with FINS. D: CK-18 was positively correlated with HOMA-IR. E: CK-18 was positively correlated with TG. F: CK-18 was positively correlated with TC. G: CK-18 was positively correlated with HDL. H: CK-18 was positively correlated with LDL.

CD3+ is a mature total T lymphocyte, CD4+ is a helper T cell and CD8+ is a toxic T cell. The liver is an important immune organ, in which T lymphocyte subsets are very important [9]. Previous studies have shown that the CD4+ memory T cell subset is an important driving force for NAFLD from steatosis to fibrosis [10]. Other studies have shown that NAFLD can lead to the loss of selective CD4+ T lymphocytes and promote the occurrence of liver cancer [11]. In addition, CD4+ T cells mainly act by themselves or by regulating cytokines secreted by other immune cells, and cytokines such as interferon and interleukin secreted by CD4+ T cells can directly damage islet β cells and inhibit insulin secretion, thus inducing insulin resistance [12, 13]. These explorations indicated that T lymphocyte subsets act in the development of T2DM and NAFLD. Cytokeratin 18 (CK-18) is an intracellular protein, which is mainly sourced form necrotic and apoptotic cells of epithelial origin [14]. CK-18 is abnormally elevated in NAFLD patients, and it is considered to be acted in promoting the development of NAFLD [15, 16]. As far as we know, there are few studies to discuss the levels of CD3+, CD4+ and CK-18 in elderly T2DM patients with NAFLD, and few to explore their correlation with NAFLD and glucose and lipid metabolism.

In this research, CD3+, CD4+ and CK-18 in elderly T2DM patients with NAFLD and their correlation with glucose and lipid metabolism were investigated, and the diagnostic efficacy of CK-18, CD3+ and CD4+ levels in elderly T2DM patients with NAFLD was tested.

Materials and methods

Research participants

Altogether 108 patients with T2DM hospitalized in Geriatrics, Taizhou People's Hospital

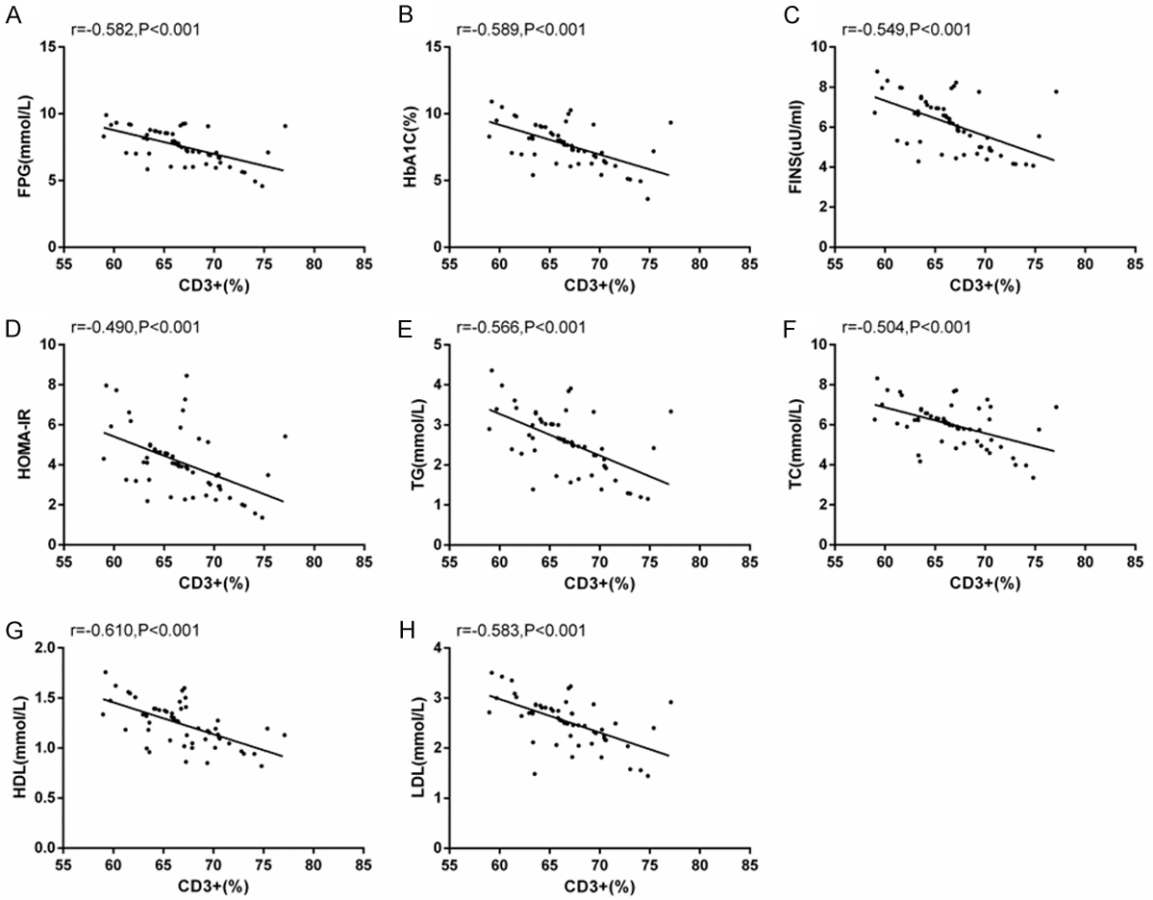

Figure 4. Correlation of CD3+ with glucose and lipid metabolism. A: CD3+ was negatively correlated with FPG. B: CD3+ was negatively correlated with HbA1C. C: CD3+ was negatively correlated with FINS. D: CD3+ was negatively correlated with HOMA-IR. E: CD3+ was negatively correlated with TG. F: CD3+ was negatively correlated with TC. G: CD3+ was negatively correlated with HDL. H: CD3+ was negatively correlated with LDL.

from August 2019 to December 2020 were obtained and grouped into' a Non-NAFLD group (58 cases) and NAFLD group (50 cases) according to the patients' conditions. Inclusion criteria: the research conformed to the diagnostic criteria of T2DM in 1999 [17], and patients aged ≥ 60 years. Exclusion criteria: patients with severe infection and acute and chronic complications of diabetes; patients have applied diuretics, beta blockers, lipid-lowering drugs, hormones or other drugs affecting glucose metabolism and lipid metabolism within 2 weeks before enrollment; patients with a history of long-term drinking (the amount of alcohol is ≥ 40 g per week); patients with viral hepatitis, drug-induced liver disease, Wilson disease, total parenteral nutrition, autoimmune liver disease, various liver cirrhosis, biliary obstruction or other diseases. Another 50 healthy people were obtained as the control group (CG). This study met the Ethics Committee, and all subjects agreed with the testing.

Treatment and serum specimen collection

Two groups of patients were given symptomatic treatment after admission. The operation was as follows: patients were asked to keep a low calorie diet and guided to get proper exercise. In addition, patients were given metformin hydrochloride tablets orally on an empty stomach, with an initial dose of 0.25 g/time, twice a day. After one week of medication, if the patient's condition was not well controlled, the dosage will be adjusted to 0.5 g/time, 3 times/d for 8 weeks.

5 ml of peripheral venous blood was obtained before treatment and after treatment, and 5 ml of blood was also obtained from the healthy

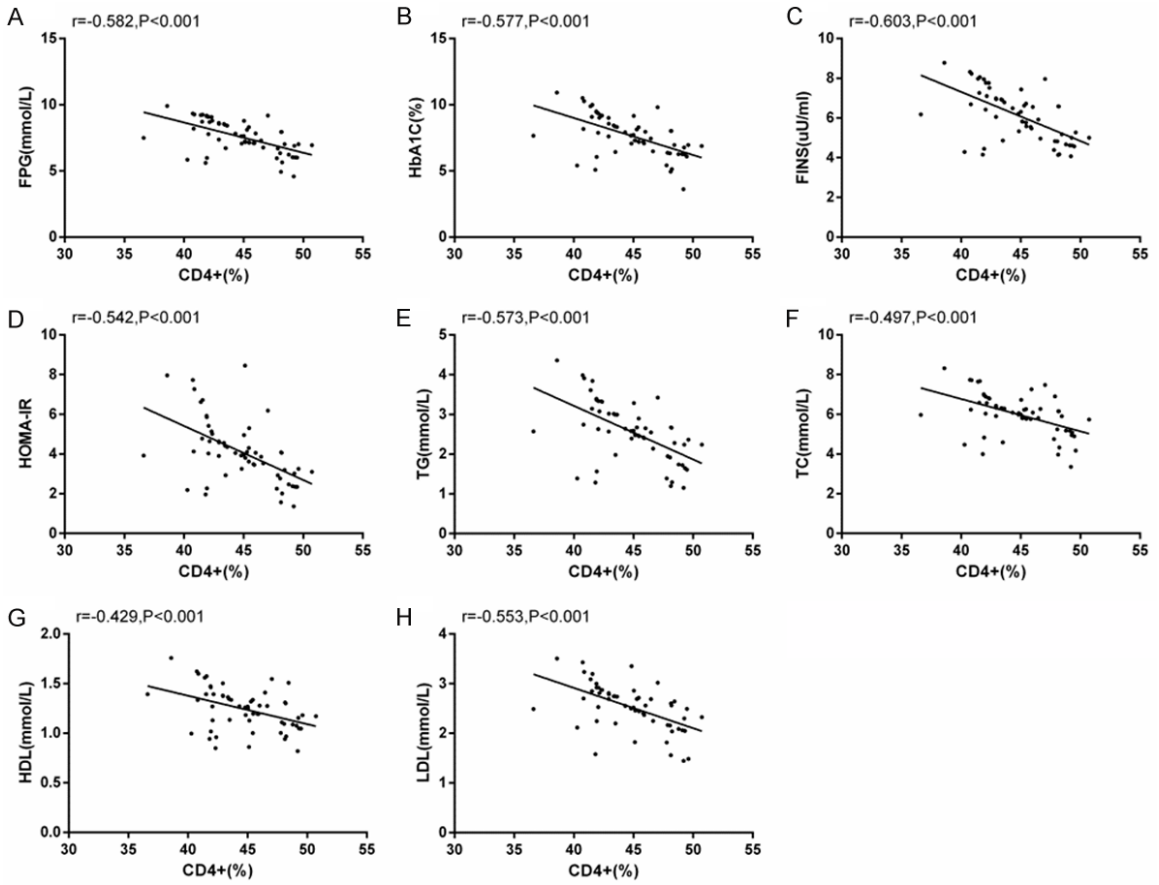

Figure 5. Correlation of CD4+ with glucose and lipid metabolism. A: CD4+ was negatively correlated with FPG. B: CD4+ was negatively correlated with HbA1C. C: CD4+ was negatively correlated with FINS. D: CD4+ was negatively correlated with HOMA-IR. E: CD4+ was negatively correlated with TG. F: CD4+ was negatively correlated with TC. G: CD4+ was negatively correlated with HDL. H: CD4+ was negatively correlated with LDL.

controls in the morning of the physical examination. The blood was centrifuged at 1500×g and at 4°C. After 10 min, the serum was separated, packaged in EP tube and stored in a freezer at -80°C.

Index testing

Biochemical indexes such as liver and kidney function [alanine aminotransferase (ALT), aspartate aminotransferase (AST)], blood lipid [triglyceride (TG), cholesterol (TC), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL)] were tested using automatic biochemical analyzer. Fasting plasma glucose (FPG) was tested. Glycosylated hemoglobin (HbA1c) was tested using micro resin exchange chromatography. Fasting insulin (FINS) was tested using luminescence immunoassay [Insulin resistance index (HOMA-IR) = (FPG) × (FINS)/22.5]. The height, weight and waist (horizontally from the lower edge of the costal arch to the midpoint between the anterior superior iliac spine) were tested, and the number was corrected to one decimal place [BMI = weight (Kg)/height $(m)^2$].

T cell subsets (CD3/CD4) were monitored using flow cytometry with monoclonal immunofluorescence and tested using direct fluorescence staining. Serum CK-18 fragment was tested using ELISA double antibody sandwich method. The steps were operated according to the kit instructions (Wuhan BOSTER Biotechnology Co., Ltd.).

Statistical methods

SPSS 21.0 was applied for statistical analysis, and GraphPad Prism 7 for visualizing the data. Chi-square test was applied for counting data comparison, independent t-test for measurement data comparison, one-way ANOVA for multiple-group mean value comparison, Dun-

Table 2. Diagnostic value of CK-18, CD3+, and CD4+ levels in elderly T2DM patients with NAFLD

Group	AUC	95% CI	Standard error	Cut-off value	Sensitivity (%)	Specificity (%)
CK-18	0.875	0.810-0.940	0.033	11.09 (ng/L)	72.00	87.93
CD3+	0.867	0.801-0.934	0.034	65.95 (%)	88.00	72.41
CD4+	0.871	0.807-0.935	0.033	44.08 (%)	92.00	68.97

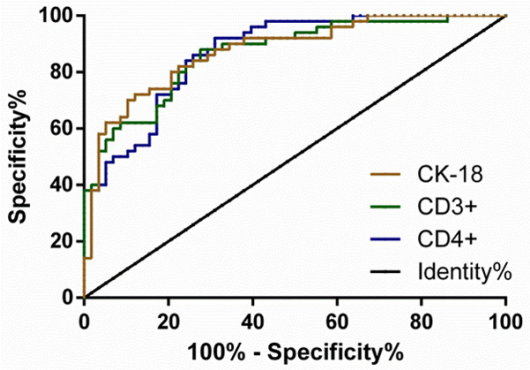

Figure 6. ROC curve of CK-18, CD3+, and CD4+ for diagnosis of elderly T2DM patients with NAFLD.

nett-t-test for pairwise comparison, and paired t-test for comparison before and after treatment. Elderly T2DM patients with NAFLD were applied as dependent variables and FINS, HOMA-IR, ALT, TG, CK-18, CD3+, and CD4+ as independent variables. Logistic regression was applied to test the factors affecting elderly T2DM patients with NAFLD. $P < 0.05$ was statistically significant.

Results

Comparison of CK-18, CD3+ and CD4+ in three groups

Compared with the CG, CK-18 in the other two groups was evidently elevated, and CK-18 in NAFLD group was elevated compared to the Non-NAFLD group ($P < 0.05$). Compared with the CG, CD3+ and CD4+ in the other two groups were decreased, and CD3+ and CD4+ in NAFLD group were decreased compared to Non-NAFLD group ($P < 0.05$) (**Figure 1**).

CK-18, CD3+, and CD4+ before and after treatment

CK-18, CD3+ and CD4+ were tested. After treatment, CK-18 was elevated, while CD3+ and CD4+ declined ($P < 0.05$). CK-18 in the NAFLD group was elevated compared to the Non-NAFLD group, while CD3+, and CD4+ were decreased compared to the Non-NAFLD group ($P < 0.05$) (**Figure 2**).

Comparison of biochemical indexes among the three groups

Compared with the CG, FPG, HbA1C, FINS, HOMA-IR, ALT, AST, TG, TC, LDL and BMI in the Non-NAFLD and NAFLD group were elevated ($P < 0.05$). Compared with Non-NAFLD group, FINS, HOMA-IR, ALT, TG and TC in NAFLD were elevated ($P < 0.05$) (**Table 1**).

Correlation of CK-18 with glucose and lipid metabolism

Pearson analysis showed that CK-18 had a positive correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL, and LDL ($P < 0.05$) (**Figure 3**).

Correlation of CD3+ with glucose and lipid metabolism

Pearson analysis showed that CD3+ had a negative correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL, and LDL ($P < 0.05$) (**Figure 4**).

Correlation of CD4+ with glucose and lipid metabolism

Pearson analysis showed that CD4+ had a negative correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL and LDL ($P < 0.05$) (**Figure 5**).

Role of CK-18, CD3+, and CD4+ in elderly T2DM patients with NAFLD

ROC curve of CK-18, CD3+, CD4+ for the diagnosis of elderly T2DM patients with NAFLD showed that the AUC value of CK-18 was 0.875, the sensitivity was 72.00%, and the specificity was 87.93%. Those of CD3+ were 0.867, 88.00%, and 72.41%, while those of CD4+ were 0.871, 92.00%, and 68.97% (**Table 2** and **Figure 6**).

Analysis of risk factors of NAFLD in elderly T2DM patients

In **Table 1**, biochemical indexes (FINS, HOMA-IR, ALT, TG, CK-18, CD3+, CD4+) with statistical significance in Non-NAFLD and NAFLD groups were taken as independent variables,

Table 3. Analysis of risk factors for NAFLD in elderly T2DM patients

Variable	β	Wals	P	OR	95% CI
FINS	0.835	4.124	0.034	1.158	0.105-1.684
HOMA-IR	1.125	7.656	0.005	2.872	1.235-5.396
ALT	0.126	5.142	0.125	2.421	1.215-2.014
TG	1.305	2.072	0.086	1.026	0.879-2.125
CK-18	1.054	6.548	0.011	2.118	1.351-4.762
CD3+	0.862	4.315	0.027	1.253	1.101-1.986
CD4+	0.792	3.862	0.035	1.268	1.076-2.021

and elderly T2DM patients with NAFLD were taken as dependent variables, and Logistic regression was conducted. The exploration revealed that FINS, HOMA-IR, CK-18, CD3+ and CD4+ were all related factors leading to NAFLD in elderly T2DM patients (**Table 3**).

Discussion

With changes in people's living habits, T2DM and NAFLD have become common diseases all over the world, which seriously affect the life and health of patients. These two diseases can influence each other and progress together, thus increasing the difficulty of disease management and resulting in worse prognosis [18]. Therefore, the exploration on the correlation of T2DM with NAFLD is of current research interest.

The T lymphocyte is the main immune cell group of cellular immunity, and they interact and antagonize each other in normal organisms, thus maintaining the balance of normal immune response [19]. In T lymphocyte subsets, CD3+ is the mature total T lymphocyte and CD4+ is the helper T lymphocyte. Under normal conditions, the absolute value and percentage of CD3+ and CD4+ are often constant, and when the immune balance of the body is perturbed, they will become abnormal [20, 21]. Compared with the CG, CD3+, and CD4+ in the other two groups were decreased, and CD3+ and CD4+ in NAFLD group were decreased compared to those in the Non-NAFLD group. In addition, after symptomatic treatment, CD3+ and CD4+ were increased. This suggested that the change in T cell subsets affected the development of T2DM and NAFLD. Lipid synthesis in liver is closely related to glucose metabolism. When glycogen storage and energy demand are normal, glucose can be converted to lipid production. Because of insulin resistance or insulin deficiency in diabetic adipose tissue, lipase activity in adipose tissue is elevated, and neutral fat is decomposed into free fatty acids and released into the liver, thus increasing the risk of NAFLD [22, 23]. Previous studies have found that invariant natural killer T cells (iNKT) are enriched in mammalian adipose tissue, and can prevent diet-induced obesity and metabolic disorders by regulating the production of cytokines [24]. Other studies have found that deleting CD8+ T cells alone can reduce inflammation and metabolic abnormalities caused by obesity [25]. These explorations suggested that immune function is closely correlated with metabolism. Pearson analysis revealed that both CD3+ and CD4+ had a negative correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL, and LDL. This suggested that changes in the T cell subsets may participate in the development of T2DM and NAFLD by affecting the normal glucose and lipid metabolism of the body.

CK-18 has been considered as a representative marker of NAFLD. At present, although the correlation of CK-18 with T2DM is not clear, some studies have found that CK-18 is elevated in the serum of T2DM patients, while the CK-18 level is elevated in patients with T2DM and NAFLD, and its expression is closely related to many metabolic risk factors [26, 27]. Compared with the CG, CK-18 in the other two groups was elevated, and CK-18 in NAFLD was elevated compared to Non-NAFLD. This result was similar to the above. In addition, CK-18 decreased after symptomatic treatment. This indicated that CK-18 was correlated with the progression of T2DM and NAFLD. Then, we investigated the correlation of CK-18 with glucose and lipid metabolism indexes, and found that CK-18 had a positive correlation with FPG, HbA1C, FINS, HOMA-IR, TG, TC, HDL and LDL. This suggested that CK-18 may also participate in the development of T2DM and NAFLD by affecting normal glucose and lipid metabolism.

NAFLD can seriously threaten the life and health of patients. At present, the diagnosis of NAFLD still uses pathologic liver biopsy results as the gold standard. However, this method is invasive and expensive, and the diagnostic results may be biased due to sampling site, so there are some limitations [28]. Therefore, it is a hot spot of clinical research to seek noninvasive and reproducible diagnostic indicators for

early diagnosis of NAFLD. In this research, the diagnostic efficacy of CD3+, CD4+, and CK-18 in elderly T2DM patients with NAFLD was tested. The results showed that the AUC value of CK-18 was 0.875, the sensitivity was 72.00%, and the specificity was 87.93%, those of CD3+ were 0.867, 88.00%, and 72.41%, and those of CD4+ were 0.871, 92.00%, and 68.97%. Therefore, CD3+, CD4+, and CK-18 can be used as biologic diagnostic indicators of NAFLD in elderly T2DM patients. At the end of this study, Logistic regression revealed that FINS, HOMA-IR, CK-18, CD3+, and CD4+ were all related factors leading to NAFLD in elderly T2DM patients. For elderly T2DM patients with these high-risk recurrence factors, enough attention and targeted preventive measures should be taken, and such patients should be regularly reexamined, so as to reduce the risk of NAFLD.

To sum up, CD3+, CD4+, CK-18 in elderly T2DM patients with NAFLD were evidently correlated with glucose and lipid metabolism, and those indexes are also correlated with the development of T2DM and NAFLD and can be used as diagnostic indicators in elderly T2DM patients with NAFLD.

Disclosure of conflict of interest

None.

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