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Effects of endoplasmic reticulum stress, liver function, insulin resistance and vascular endothelial function in patients with nonalcoholic fatty liver disease

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ABSTRACT

It is aimed to compare whether there are differences in endoplasmic reticulum stress, liver function, insulin resistance, and vascular endothelial function in patients with nonalcoholic fatty liver disease (NAFLD) with and without type 2 diabetes mellitus (T2DM). Forty patients with NAFLD, 38 patients with NAFLD combined with T2DM, and 30 patients with normal liver tissue were selected. They were set as Group A, Group B and Group C respectively. The expression level of glucose-regulated protein 94 (GRP94) and biochemical indicators such as alanine aminotransferase (ALT), free fatty acids (FFA), triglycerides (TG), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), fasting blood glucose (FBG), and fasting insulin (FINS) were measured. Also, the calculation of the homeostatic model assessment for insulin resistance (HOMA-IR) index was performed. The reactive hyperemia index (RHI) was detected to evaluate the vascular endothelial function of patients. The comparison between groups and multi-factor analysis of the influencing factors of RHI was conducted. Compared with Group C, the expressions in Group A and Group B were distinctly enhanced ($P < 0.05$). Also, the expression of GRP94 protein in Group B was distinctly higher than that in Group A ($P < 0.05$). The average optical density values of Groups A, B, and C were 0.327 ± 0.007, 0.350 ± 0.009, and 0.299 ± 0.006, respectively. A comparison between the three groups was performed. The differences had statistical significance ($P < 0.05$). The differences in the TG, ALT, AST, GGT, FINS and HOMA-IR between Group A and Group B had statistical significance ($P < 0.05$). The RHI values of Groups A, B, and C were 1.59 ± 0.23, 1.79 ± 0.32, and 2.05 ± 0.47, respectively. A comparison between the three groups was performed. The differences had statistical significance ($P < 0.05$). FFA, ALT and FBG in patients with NAFLD are risk factors for endothelial dysfunction ($P < 0.05$). The liver damage caused by NAFLD may be related to the expression of GRP94. FFA, ALT and FBG are risk factors for endothelial dysfunction in NAFLD patients.

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Introduction

Currently, with the adjustment of diet structure and lifestyle, the incidence of nonalcoholic fatty liver disease (NAFLD) has been increasing. At present, it has become a new health problem that many countries are facing, chronic liver disease and a coexisting factor of other diseases that are widespread all over the world (1). There is simple fatty liver, steatohepatitis, liver fibrosis and cirrhosis in the pathological spectrum of NAFLD. Among them, 20% of patients can develop cirrhosis, 30% to 40% of patients die of liver-related diseases, and some patients develop subacute liver failure and liver cancer (2, 3). Investigations have shown that NAFLD is the primary cause of cryptogenic transaminase elevation (4).

The incidence of NAFLD is closely related to endoplasmic reticulum stress (ERS), liver function, insulin resistance (IR), and vascular endothelial function. But its exact pathogenesis is complicated, and there is no unified conclusion. There are plenty of endoplasmic reticulum in liver cells, which is an important place for protein synthesis, folding, transportation and storage of calcium. Also, it is greatly sensitive to various stimuli. When the function is disturbed, unfolded protein and misfolded protein accumulate in the cavity. The intracellular calcium balance is in a disordered state, which is ERS (5). The investigation by Wang *et al*. (2019) has shown that ERS acts a part in the pathophysiology of alcoholic liver injury (6). The pathological manifestations of alcoholic fatty liver and NAFLD are almost the same. It is speculated

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that ERS is also involved in the formation of NAFLD. Also, there is a close relationship between ERS and the development of IR. ERS in the liver may exacerbate IR and form extrahepatic tissue IR (7). Some investigations have shown that the body's IR, glucose and lipid metabolism disorder is one of the key links in the formation of NAFLD. IR can cause hepatic cell steatosis by promoting peripheral lipolysis and hyperinsulinemia (8). IR is the common mechanism of NAFLD and type 2 diabetes mellitus (T2DM). In patients with NAFLD combined with T2DM, IR performance is particularly prominent (9, 10). It can be seen that the incidence of NAFLD is closely related to risk factors such as T2DM. During the progression of NAFLD, 22% of patients developed diabetes and 23% of patients developed dyslipidemia (11, 12). The elimination of intrahepatic fat can improve IR. Therefore, NAFLD patients with IR can reduce the incidence of diabetes by decreasing the amount of fat accumulation (13, 14). Scholars such as Choi *et al*. (2018) suggested that abnormal liver function index levels can predict the occurrence of T2DM (15). In addition, there is a close connection between the prognosis of patients with NAFLD and the occurrence of cardiovascular and cerebrovascular diseases. NAFLD can develop from simple fatty liver to non-alcoholic steatohepatitis and induce arteriosclerotic cardio-cerebrovascular events by exacerbating IR. It has been regarded as an independent disease (16). Loffredo *et al*. (2018) found that in NAFLD patients, flow-mediated vasodilatation (FMV), which reflects vascular endothelial function, decreased, and the severity of NAFLD was significantly associated with low FMV. That is, the FMV value of patients with non-alcoholic steatohepatitis is lower than that of patients with simple fatty liver and even lower than those with normal liver tissue (17).

To further explore the pathogenesis of NAFLD, the experiment selected NAFLD patients and NAFLD combined with T2DM patients as the research subjects to explore the effects of ERS, liver function, insulin resistance, and vascular endothelial function in NAFLD patients. Then, a theoretical basis will be provided for the clinical prevention and treatment of NAFLD.

Materials and methods

Research Subject

40 NAFLD patients diagnosed by history, clinical manifestations and auxiliary examinations at XXX Hospital (December 2017-December 2019) and 38 patients with NAFLD combined with T2DM were selected. In addition, 30 patients with normal liver tissues matched by gender and age were selected as the control. They were set as Groups A, B and C. The research was reviewed and approved by the hospital ethics committee for implementation. The relevant informed consent was signed by all patients and their families.

Inclusion criteria: the diagnosis of NAFLD is based on the diagnostic criteria established by the Fatty Liver and Alcoholic Liver Diseases Group of the Hepatology Branch of the Chinese Medical Association; the diagnosis of T2DM complies with the diagnostic criteria for diabetes published by the World Health Organization; patients with perfect clinical data.

Exclusion criteria: drug-induced liver damage, viral hepatitis, total parenteral nutrition, autoimmune liver disease, alcoholic liver disease, and other specific diseases that can cause liver dysfunction or fatty liver; patients with cirrhosis; liver cancer patients; other progressive fatal disease; alcoholism, pregnancy patients.

General Data Collection

All the subjects were asked detailed medical history, including age, gender, drinking history, past medical history (fatty liver, diabetes, hypertension, dyslipidemia) and family history (fatty liver, diabetes, hypertension, dyslipidemia, obesity). The measurement of their weight, height, waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), and other data was performed. At the same time, the following calculation was performed: body mass index (BMI) = body weight (kg)/height^2 (m^2).

Measurement of the Expression Level of Glucose Regulated Protein 94 (GRP94) By Immunohistochemistry

Fatty liver tissue of NAFLD patients was obtained by laparoscopic surgery. Normal control liver tissue was obtained by surgery for metastatic colorectal

cancer. All the obtained fatty liver tissue and normal control liver tissue weighed about 0.2g. They were quickly put into a liquid nitrogen tank (Shanghai Yuyan Scientific Instrument Co., Ltd., China) to freeze, and store at -80℃. The expression level of GRP94 was measured by immunohistochemistry. The immunohistochemistry method labelled a certain enzyme on the antibody of the corresponding antigen in the specimen. The color reagent was used to make the part of the antigen-antibody reaction in the specimen into a color observable by the naked eye under the microscope to determine whether the antigen to be tested is present.

The liver tissue was fixed, embedded in paraffin, and sliced continuously with a thickness of 4μm. Also, it was spread on the anti-off slide, and put in a 37℃ incubator to dry. After dewaxing and hydration with xylene and ethanol, it was washed with phosphate buffer saline (PBS) (Tianjin Guangcheng Chemical Reagent Co., Ltd., China) for 3×5 minutes. 3% H_2O_2 was added to inactivate endogenous catalase. It was blocked at room temperature for 10 minutes and washed with distilled water 3 times. It was put in 0.01M citric acid buffer (pH 6.0, Xiamen Yanke Biotechnology Co., Ltd., China), to restore the antigen in the autoclave for 8 minutes. After removing and cooling, it was washed with PBS for 3×5 minutes. The normal goat serum blocking solution (Anhui Jingke Biotechnology Co., Ltd., China) was added. After 20 minutes, the rabbit anti-human GRP94 polyclonal antibody (dilution ratio 1:50) (Beijing Boaosen Biotechnology Co., Ltd., China) was added overnight at -4℃. After taking it out, it was rewarmed for 45 minutes and washed with PBS for 3×5 minutes. Adding secondary antibody dropwise, it was incubated in a 37℃ CO_2 incubator for 20 minutes and washed with PBS for 3×5 minutes. Adding streptomycin avidin-biotin complex, it was incubated in a 37℃ CO_2 incubator for 20 minutes and washed with PBS for 3×5 minutes. After DAB (Diaminobenzidine) color development kit (Beijing Chreagen Biotechnology Co., Ltd., China) was used for 10 minutes, it was rinsed with distilled water. After hematoxylin (Fuzhou Maixin Biotechnology Development Co., Ltd., China) was counterstained for 6 seconds, it was dehydrated and sealed. The optical microscope (Leica, Germany) was used to observe the slices and the picture was taken to save it. Under the optical microscope, 6 non-overlapping fields were randomly selected for each slice. 0.01M PBS was used instead of the primary antibody as a blank control. The average absorbance was measured using a fully automated medical color image analysis system. The average of each group was calculated to reflect the positive intensity.

Measurement of Biochemical Indicators

After fasting for 12 hours, all subjects were collected 10ml of elbow median venous blood sample in the early morning. After centrifuging the plasma, and the DXC800 automatic biochemical analyzer (Beckman Coulter, USA) was immediately used to measure the following biochemical indicators: blood lipid indicators such as glycated hemoglobin 1C (HbA1C), total cholesterol (TC), free fatty acid (FFA), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C); liver function indicators such as alanine transaminase (ALT), gamma-glutamyl transferase (GGT), and aspartate transaminase (AST); fasting blood glucose (FBG), and fasting insulin (FINS).

The homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated to evaluate the individual's IR level. The equation: HOMA-IR=FBG (mmol/L) × FINS (μU/mL)/22.5. At the same time, by calculating the homeostatic model assessment of β-cell function (HOMA-β) index, the individual's β-cell function was evaluated. The equation: HOMA-β=20×FINS/(FBG-3.5).

Noninvasive Endothelial Function Test

All patients must not consume coffee or smoke within 12 hours before the test. At the same time, it is necessary to avoid influencing factors such as staying up late, menstrual cycle, antioxidant treatment and cysteine. The patient needed to remain quiet for 30 minutes before the examination to keep the spirit in a soothing state. The examination should be performed on an empty stomach or more than 2 hours after a meal. The patient took a supine position and relaxed the whole body. The test operation was responsible for the specific professional technicians who have been systematically trained. Endo-PAT2000 noninvasive vascular endothelial function tester (Itamar, Israel) was used to test the vasodilation function of the index finger. The inflatable cuff was put on the patient's right upper arm. The two-finger

sleeve probes were respectively worn on the index fingers of the two hands of the patient while not touching other fingers. One side monitored systemic vascular changes as a control, and the other side tested vascular endothelial function. Then, the automatic path of the instrument was used to inflate and pressurize the probe finger cuff. The sensor located in the probe automatically inputs the blood flow signal (BFS) of the arterial vascular bed of the tested finger end into the computer software. After the BFS of the vascular bed of the finger end tended to be stable, the vasodilation function of the index finger was measured. The BFS data of the finger end was collected for 6 minutes as the reference. The cuff was quickly inflated to block the blood flow of the brachial artery until the pressure was maintained at 200mmHg. At this time, the software showed the disappearance of the BFS on one side of the finger end. After continuing to record for 5 minutes, it was quickly deflated to adjust the pressure to zero. The blocked BFS was restored, continuing to record for 5 minutes. After the test operation was completed, the ratio of the signal amplitude before and after blocking was calculated by the Endo-PAT system software. The contralateral data was used as a control to modify the test results. Then, the reactive hyperemia index (RHI), an indicator of vascular endothelial cell function, was calculated. The normal value of RHI should be larger than 1.67. The higher the value, the better the endothelial function, otherwise, the endothelial function decreases.

Statistical Analysis

The experiment used SPSS26.0 statistical software to analyze data. The mean ± standard deviation ($\bar{x}\pm s$) was used to express the measurement data. The experiment adopted the t-test to compare the means of the two samples. Counting data was expressed in incidence n (%). The x^2 test was adopted for comparison. The multivariate analysis was performed by the binomial logistic multiple regression analysis. The difference had statistical significance with P <0.05.

Results and discussion

General Clinical Data of Three Groups of Patients

The general clinical data of the three groups are shown in Table 1. Patients in Group A were 27 males and 13 females, aged 50.12 ± 10.49 years old. Patients in Group B were 25 males and 13 females, aged 50.78 ± 11.07 years old. Patients in Group C were 19 males and 11 females, aged 49.43 ± 10.32 years old. Comparing the basic data such as age and gender of the three groups A, B, and C, the difference had no statistical significance (P> 0.05). The investigations were comparable. The BMI, waist circumference, SBP and DBP of Group A were distinctly lower than those of Group B and higher than Group C. The differences had statistical significance (P <0.05).

Table 1. General clinical data of three groups

General clinical data	Group A (n = 40)	Group B (n = 38)	Group C (n = 30)
Age (year)	50.12 ± 10.49	50.78 ± 11.07	49.43 ± 10.32
Gender (male/female, case)	27/13	25/13	19/11
BMI (kg/m^2)	23.34 ± 2.01^a	26.17 ± 2.37^{ab}	22.56 ± 1.55
Waist circumference (cm)	84.01 ± 6.69^a	96.01 ± 7.15^{ab}	73.66 ± 5.87
SBP (mmHg)	130.31 ± 13.65^a	136.76 ± 14.98^a	115.92 ± 13.35
DBP (mmHg)	77.67 ± 8.02^a	82.25 ± 9.12^a	72.89 ± 7.54

Note: a: compared to Group C, P <0.05; b: compared to Group A, P <0.05

Test Results of Immunohistochemistry

The results of immunohistochemistry are shown in Figure 1. GRP94 protein staining was located in the cytoplasm of hepatocytes. In the liver tissues of patients in Groups A and B, rich tan particles can be tested. Compared with Group A, the expression of Group B was distinctly enhanced, and the difference had statistical significance (P <0.05). In Group C, light yellow particles scattered in the cytoplasm of hepatocytes can be seen. Compared with Group C, the expressions in Groups A and B were distinctly enhanced, and the difference had statistical significance (P <0.05). The results of the average optical density of the three groups are shown in Figure 2. The average optical densities of Groups A, B, and C were 0.327 ± 0.007, 0.350 ± 0.009, and 0.299 ± 0.006. The comparison between the three groups was performed and the differences had statistical significance (P <0.05).

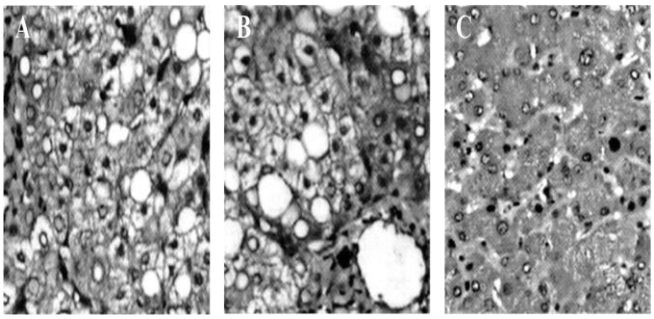

Figure 1. Test results of immunohistochemistry. A. GRP94 protein expression in Group A (×100). B. GRP94 protein expression in Group B (×100). C. GRP94 protein expression in Group C (×100)

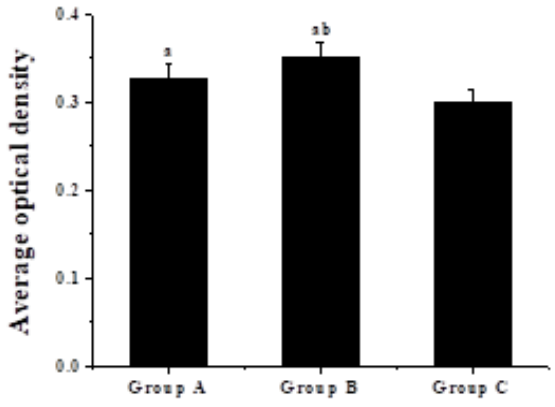

Figure 2. The average optical density of the three groups (Note: a: compared to Group C, P <0.05; b: compared to Group A, P <0.05)

Test Results of Biochemical Indicators

The test results of biochemical indicators are shown in Figure 3. Comparing HbA1C, FFA, TC, HDL-C, LDL-C, FBG and HOMA-β in Groups A and B with those in Group C, the differences had statistical significance (P <0.05). However, there was no significant difference in HbA1C, TC, FFA, HDL-C, LDL-C, FBG and HOMA-β between Group A and Group B (P> 0.05). The TG and HOMA-IR of Group A were distinctly lower than those of Group B and higher than Group C. The differences had statistical significance (P <0.05). Compared with Groups A and C, ALT, AST, GGT and FINS in Group B were distinctly increased, and the differences had statistical significance (P <0.05).

Test Results of Noninvasive Endothelial Function

The test results of noninvasive endothelial function are shown in Figure 4. The RHI value of patients in Group A was 1.59 ± 0.23, and the RHI value of patients in Group B was 1.79 ± 0.32, and the RHI value of patients in Group C was 2.05 ± 0.47. Compared with Group C, the RHI values of patients in Groups A and B were distinctly reduced, and the difference had statistical significance (P <0.05). Compared with Group A, the RHI value of patients in Group B was distinctly increased, and the difference had statistical significance (P <0.05).

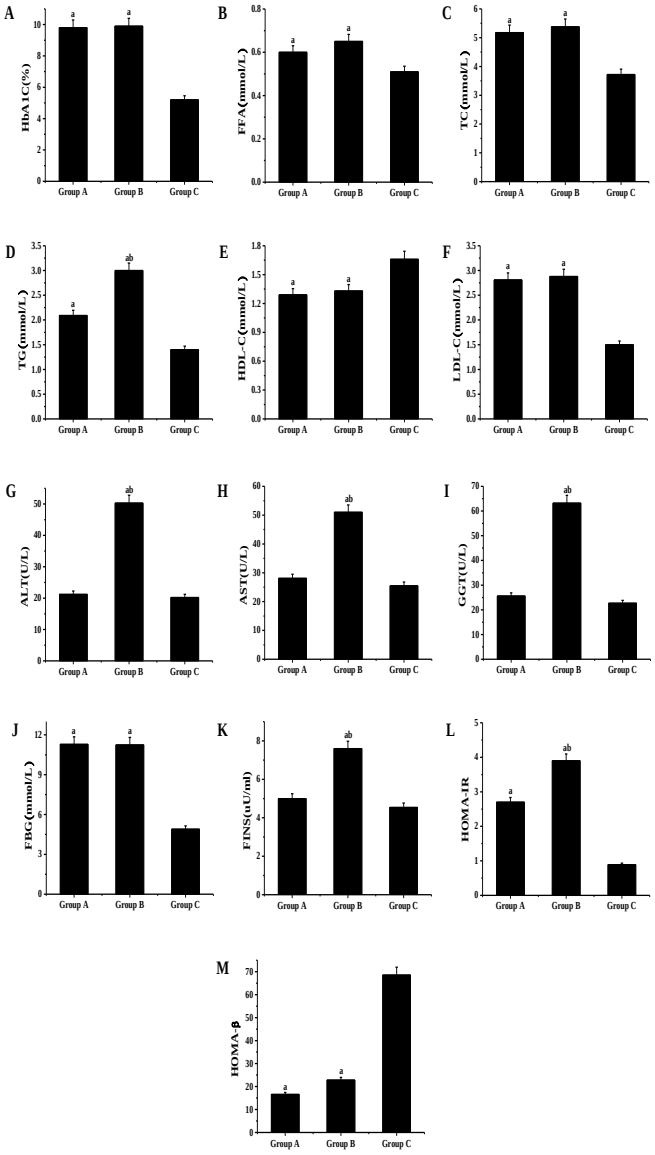

Figure 3. Test results of biochemical indicators (Note: a: compared to Group C, P <0.05; b: compared to Group A, P <0.05) A. HbA1C. B. FFA. C.TC. D. TG.E.HDL-C. F. LDL-C. G. ALT. H. AST. I. GGT. J. FBG. K. FINS. L. HOMA-IR. M. HOMA-β.

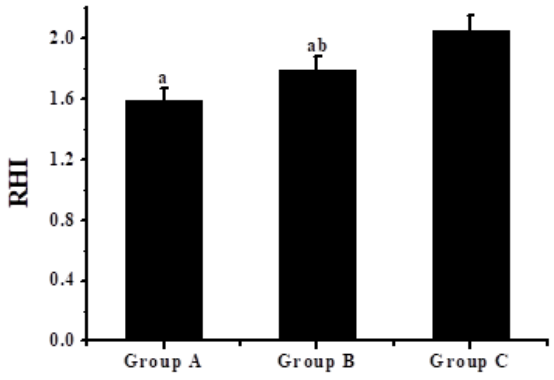

Figure 4. Test results of noninvasive endothelial function

Results of Correlation Analysis Between RHI and Clinical Indicators in NAFLD Patients

The results of multivariate unconditional Logistic regression analysis of endothelial dysfunction in NAFLD patients are shown in Table 2. The dependent variable was whether the endothelial function was damaged (Yes = 1, No = 0). Other clinical indicators were taken as independent variables. The multivariate unconditional Logistic regression analysis was conducted. The results showed that FFA, ALT and FBG in NAFLD patients were risk factors for vascular endothelial dysfunction (P <0.05).

Table 2. Results of multivariate unconditional Logistic regression analysis of endothelial dysfunction in NAFLD patients

Logistic multiple regression analysis	FFA	ALT	FBG
Regression coefficients	1.726	0.043	0.030
Standard error	0.833	0.020	0.141
Odds ratio	5.594	1.035	1.345
95% confidence interval	(1.099 ~ 27.984)	(1.010 ~ 1.088)	(1.030 ~ 1.762)
x^2	4.489	5.257	4.631
P	0.040	0.019	0.028

GRPs are a kind of endoplasmic reticulum molecular chaperone and a type of stress protein produced by cells to adapt to the ERS state (18). Among them, GRP94 is the most abundant glycoprotein in the endoplasmic reticulum, only found in eukaryotes (19). GRP94 is a highly conserved and monomorphic glycoprotein that can bind calcium ions tightly. It also has magnesium ion-dependent ATPase activity. The unfolded polypeptide chains can be combined to prevent protein accumulation, help protein folding, stretching, assembly and transport, thereby preventing the secretion of misfolded proteins (20-22). Therefore, GRP94 is also an important marker of ERS and acts a part in lipid and protein metabolism. In the investigation, the expression level of GRP94 was tested by immunohistochemistry to explore the role of ERS in NAFLD to further clarify its pathogenesis. It was found that the expression level of GRP94 protein in the liver tissue of NAFLD patients was significantly enhanced. GRP94 was considered to be involved in the formation of NAFLD. It is consistent with the original speculation that ERS is involved in the formation of NAFLD. The experiment reached expectations.

The main physiological function of the vascular endothelium is achieved through its permeability barrier function for material exchange and active transport inside and outside the blood vessel (23). The severity of vascular endothelial dysfunction can dynamically reflect the progress of atherosclerosis (AS) and is used to predict the outcome of AS disease (24). In this investigation, the noninvasive vascular endothelial function was used to test the RHI value to evaluate the vascular endothelial cell function of patients. The results showed that the RHI value of NAFLD patients was slightly lower than the reference value of the normal population, suggesting a trend of endothelial dysfunction. Compared with Group A, the RHI value of patients in Group B was distinctly increased (P <0.05), suggesting that the endothelial function of patients with NAFLD alone was lower than that with T2DM. Therefore, in patients with NAFLD combined with T2DM stage, if the RHI value changes differently, it indicates that the early compensatory filling function of endothelial dysfunction declines. Scholars such as Taylor *et al*. (2018) investigated patients with T2DM. It was found that excluding other factors, only considering the slightly elevated transaminase level caused by NAFLD was independently associated with mediated vasodilation of low brachial artery endothelial blood flow and high insulin sensitivity (25). It confirmed that NAFLD can affect the formation of early AS, which is consistent with the results of this investigation. Through multivariate analysis, FFA in patients with NAFLD is a risk factor for vascular

endothelial dysfunction (P <0.05). Long-term hyper-FFA can lead to lipotoxicity, damage β cells, increase IR, and damage the activity of endothelial nitric oxide synthase, resulting in reduced nitric oxide production. As a result, normal vasoconstriction is impaired (26). This investigation also found that ALT is a risk factor for endothelial dysfunction in NAFLD patients. The investigation by Wang *et al*. (2019) has shown that high ALT levels in NAFLD patients reflect high liver inflammation and are closely related to endothelial dysfunction (27). It is consistent with the results of this investigation. In addition, FBG is also one of the risk factors for endothelial dysfunction. Hepatic steatosis can also promote liver IR by affecting the insulin signaling pathway, resulting in T2DM (28). It can be seen that T2DM patients with unstable blood sugar control can stabilize blood sugar by reducing liver fat content and liver inflammation. Therefore, in the early stage of the NAFLD, the endothelial function impairment can be delayed by blocking high-risk factors such as FFA, ALT, and FBG, so that the development of AS can be reduced or delayed to improve the prognosis of patients.

In summary, GRP94 is expressed in NAFLD, indicating that ERS acts a part in the occurrence and development of NAFLD. Moreover, NAFLD patients tend to have endothelial dysfunction. Among them, FFA, ALT and FBG are risk factors for endothelial dysfunction. The investigation provides a reference for the clinical prevention and treatment of NAFLD, with great significance. However, there are some shortcomings in this process. The small amount of data collected by the sample leads to a certain degree of deviation in the results. Thus, the data capacity will be further enhanced in the later period, so that the obtained results are more valuable.

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None.

Interest conflict

The authors declare no conflict of interest.

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