Glucagon is associated with NAFLD inflammatory progression in type 2 diabetes, not with NAFLD fibrotic progression

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Objectives Higher prevalence of progressive stages of nonalcoholic fatty liver disease (NAFLD) and hyperglucagonemia were observed in type 2 diabetes. We aim to investigate whether islet alpha cell dysfunction (evaluated by glucagon) associates with NAFLD progression in type 2 diabetic adults.
Methods A total of 4937 diabetic participants were enrolled from seven communities in Shanghai, China. Probable nonalcoholic steatohepatitis (NASH) was defined by the presence of NAFLD and metabolic syndrome. Probable NAFLD fibrosis score was used to identify patients with different risk stratification of bridging fibrosis (stage 3) or cirrhosis (stage 4).
Results After adjustment for age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitor, glucagon quartiles were negatively associated with probable NASH (Q4 vs. Q1 OR 0.71, 95% confidence interval, 0.53–0.96, *P* for trend=0.010), though they were not associated with simple NAFLD (*P* for trend=0.176). Furthermore, glucagon was not significantly associated with fibrotic progression of liver steatosis in diabetic patients with NAFLD (*P* for trend=0.889).
Conclusions Significant associations were observed among glucagon and inflammatory progression of NAFLD, but not with fibrotic progression. Further understanding the association between islet alpha cell and liver may lead to development of treatment strategies for NAFLD patients with type 2 diabetes. Eur J Gastroenterol Hepatol 33: e818–e823

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases in the world [1,2]. The prevalence of NAFLD is higher in patients with type 2 diabetes mellitus (T2DM) [3]. Moreover, patients with both T2DM and NAFLD are prone to develop severe histological forms of NAFLD, including NASH, advanced fibrosis and cirrhosis [4]. NASH has now become the second leading cause for liver transplantation in the USA [5] and fibrosis is closely associated with long-term mortality [6]. Therefore, great attention should be paid to the progression of NAFLD, especially in diabetic patients.

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Keywords: advanced fibrosis, glucagon, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, type 2 diabetes mellitus

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Glucagon is a peptide hormone secreted by islet α cells, contributing to the maintenance of euglycemia in human. Elevated plasma glucagon concentration is a biochemical hallmark of diabetes [7]. Glucagon can increase hepatic glucose production during fasting state [8]. Because glucagon secretion is highly affected by insulin, it may make sense to consider the glucagon-to-insulin ratio instead of assessing absolute values [9]. Moreover, a recent study with a small sample size found the association between glucagon-to-insulin ratio and the presence of NAFLD.

Thus, we suppose glucagon may be associated with NAFLD progression including inflammation and fibrosis. Liver biopsies could not be applied in epidemiology study, so we used metabolic syndrome, one of the strong noninvasive NASH predictors [10], to assess probable NASH; and we used NAFLD fibrosis score (NFS), a noninvasive system that identifies liver fibrosis in patients with NAFLD [11], to assess probable NAFLD fibrosis. NFS is a noninvasive scoring system that accurately separates NAFLD patients with and without advanced fibrosis [11].

Therefore, in this large community-based study, we aimed to investigate whether glucagon associates with inflammatory and fibrotic progression of NAFLD evaluated by above noninvasive measurements in Chinese type 2 diabetic adults.

Materials and methods

Study design and participants

We designed a population-based study named METAL study in 2018 (Environmental Pollutant Exposure and

Metabolic Diseases in Shanghai, www.chictr.org.cn, ChiCTR1800017573). Participants of this study were enrolled from seven communities in Huangpu and Pudong District, Shanghai, China. We randomly selected patients with diabetes from the community healthcare center registration platform. Chinese citizens adults who had lived in their current area for ≥6 months were included. Totally, 4937 subjects with diabetes received the examination. Participants who were missing laboratory results (n = 8), questionnaire data (n = 116), missing glucagon data (n = 5), had a history of excessive consumption (men ≥140 g/week, women ≥70 g/week) of pure alcohol (Chinese Society of Hepatology 2010) (n = 225), was using medications associated with secondary NAFLD (corticosteroids, estrogens, amiodarone, methotrexate) (n = 157), had self-reported viral hepatitis (including hepatitis B and hepatitis C virus) (n = 243), and did not have ultrasound result (n = 68) were excluded. Finally, 4115 diabetic participants were involved in the final analyses (Fig. 1).

The study protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee. Informed consent was obtained from all participants included in the study.

Measurements

We followed the methods of Wang *et al*. [12]. The questionnaire included sociodemographic characteristics, family history, medical history and lifestyle factors. The trained and experienced personnel were the same group as those in SPECT-China study [13]. The interviews and clinical examinations were conducted according to a standard protocol. Current smoking was defined as having smoked at least 100 cigarettes in one's lifetime and currently smoking cigarettes [14]. Waist circumference was measured in the horizontal plane midway between the lowest ribs and the iliac crest, as suggested by the WHO and the International Diabetes Federation.

Blood samples were obtained between 6:00 a.m. and 9:00 a.m. after overnight fasting for at least 8 h. Blood was refrigerated immediately after phlebotomy, and it was centrifugated in 2 h and the serum was aliquoted and frozen in a central laboratory. Serum glucagon was measured by radioimmunoassay (SN-6105, Hesuorihuan, Shanghai, China). Glycated hemoglobin (HbA1c) was measured by HPLC (MQ-2000PT, Medconn, Shanghai, China). Fasting plasma glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lipid profile were performed with a Beckman Coulter AU 680 (Brea, USA).

Hypertension was assessed by systolic blood pressure ≥140 mmHg, or diastolic blood pressure ≥90 mmHg, or self-reported previous diagnosis of hypertension by physicians. Dyslipidemia was defined as total cholesterol ≥6.22 mmol/L (240 mg/dL), triglycerides ≥2.26 mmol/L (200 mg/dL), low-density lipoprotein≥4.14 mmol/L (160 mg/dL), high-density lipoprotein <1.04 mmol/L (40 mg/dL) or self-reported previous diagnosis of hyperlipidemia by physicians, according to the modified National Cholesterol Education Program-Adult Treatment Panel III.

Outcome definition

Liver fat accumulation (steatosis) was detected by ultrasound (Mindray M7, MINDRAY, Shenzhen, China) [15,16]. According to Saadeh *et al*.'s criteria, presentation of steatosis included increased liver echogenicity, stronger echoes in the hepatic parenchyma as compared to the renal parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins [17].

The presence of metabolic syndrome is a strong predictor for nonalcoholic steatohepatitis (NASH) in patients with NAFLD according to the guidelines by the American Association for the study of liver disease and the Chinese Society of Hepatology [18]. Thus, we categorized the subjects with metabolic syndrome and NAFLD into subjects with probable NASH and the left as simple NAFLD. Metabolic syndrome was diagnosed on the basis of the International Diabetes Federation criteria (2005) [19].

NFS was used to identify NAFLD patients with different risk stratification of having advanced fibrosis [18]. The formula to calculate NFS is –1.675 + 0.037*age (years) + 0.094*BMI (kg/m^2) + 1.13* impaired fasting glucose /diabetes (yes =1, no =0) + 0.99*AST/ALT ratio – 0.013*platelet (*10^9/l) – 0.66*albumin (g/dL) [11]. A score <–1.455 was defined as probable absence of advanced fibrosis (90% sensitivity and 60% specificity to exclude advanced

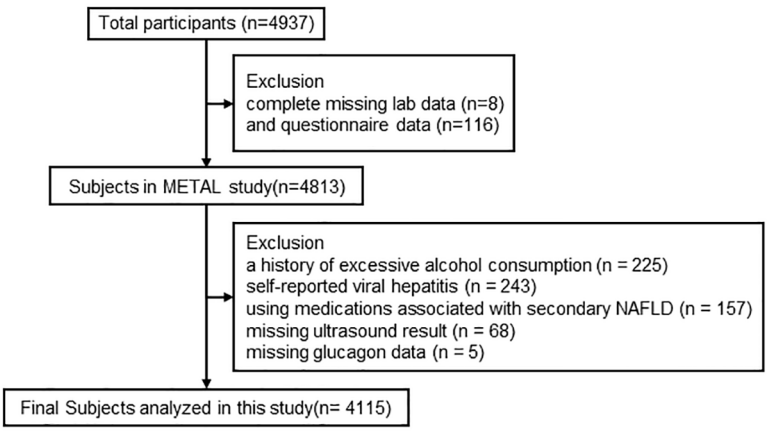

Fig. 1. Flowchart of participants' inclusion and exclusion.

Table 1. Characteristics of study participants by NAFLD category

Characteristic	Glucagon (pg/mL) Q1 (≤115.31)	Q2 (115.32–154.12)	Q3 (154.13–201.99)	Q4 (>202)	*P* for trend
N	1029	1029	1029	1028	
Age, year	67.29 ± 8.96	67.61 ± 8.88	67.84 ± 8.21	66.76 ± 8.82	0.266
Men, %	28.01	26.12	23.83	22.04	<0.001
Duration of diabetes, year	10.68 ± 8.53	9.83 ± 7.6	10.34 ± 7.97	9.97 ± 7.92	0.164
Current smoking, %	17.01	16.03	14.29	15.18	0.946
Platelet count, $*10^9$/L	210.11 ± 58.18	214.39 ± 57.75	212.39 ± 57.59	216.37 ± 57.82	0.038
Albumin, g/dL	44.48 ± 2.74	44.46 ± 2.63	44.43 ± 2.71	44.48 ± 2.68	0.930
AST/ALT	1.17 ± 0.36	1.16 ± 0.39	1.16 ± 0.42	1.17 ± 0.43	0.921
BMI, kg/m^2	24.71 ± 3.49	25.16 ± 3.73	24.99 ± 3.54	25.03 ± 3.77	0.119
Waist circumference, cm	89.55 ± 9.84	90.58 ± 9.94	90.2 ± 9.66	90.25 ± 9.59	0.210
FPG, mmol/L	7.83 ± 2.36	7.74 ± 2.34	7.72 ± 2.41	7.77 ± 2.59	0.571
HbA1c, %	7.51 ± 1.34	7.49 ± 1.4	7.48 ± 1.38	7.52 ± 1.42	0.899
HbA1c, mmol/mol	58.58 ± –8.85	58.36 ± –8.2	58.25 ± –8.42	58.69 ± –7.98	
SBP, mmHg	143.57 ± 19.6	146.33 ± 19.83	144.49 ± 19.68	145.14 ± 19.78	0.307
DBP, mmHg	78.24 ± 10.4	78.94 ± 11.18	78.54 ± 10.9	78.57 ± 10.47	0.703
Total cholesterol, mmol/L	5.03 ± 1.19	5 ± 1.19	5.17 ± 1.2	5.25 ± 1.17	<0.001
Triglycerides, mmol/L	1.83 ± 1.42	1.88 ± 1.39	1.9 ± 1.57	1.93 ± 1.45	0.119
HDL-C, mmol/L	1.18 ± 0.29	1.19 ± 0.28	1.23 ± 0.3	1.23 ± 0.3	<0.001
LDL-C, mmol/L	3.14 ± 0.85	3.08 ± 0.84	3.18 ± 0.86	3.24 ± 0.84	<0.001
Hypertension, %	76.48	83.67	76.38	78.5	0.829
Dyslipidemia, %	62.49	59.86	60.84	65.18	0.182

The data are summarized as the mean ± SD for continuous variables or as a percentage for categorical variables. *P* for trend was calculated by regression analysis. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP; diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; SBP, systolic blood pressure.

fibrosis); a score >0.676 was defined as probable presence of advanced fibrosis (67% sensitivity and 97% specificity to identify the presence of advanced fibrosis); and a score between –1.455 and 0.676 have indeterminate results [11].

Statistical analysis

Data analyses were performed using IBM SPSS Statistics, Version 21 (IBM Corporation, Armonk, New York, USA). Continuous variables were shown as mean±SD and categorical variables as percentages (%). *P* value <0.05 indicated significance (two-sided). The concentration of glucagon was logarithmically transformed to achieve a normal distribution.

Glucagon was divided into quartiles. The first quartile of glucagon (Q1) representing the lowest one and the fourth quartile (Q4) the highest. Multinomial logistic regression was used to measure the association of glucagon (independent variable) and progression category of NAFLD (dependent variable). Odds ratios (OR) with 95% confidence interval (CI) were calculated. The model was adjusted for age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitors.

Results

Characteristics of the diabetic participants by NAFLD progression

General clinical characteristics of the 4115 diabetic participants of this study by glucagon quartiles are shown in Table 1. The quartile ranges of glucagon were Q1 (≤115.31 pg/ml), Q2 (115.32–154.12 pg/mL), Q3 (154.13–201.99 pg/mL), Q4 (>202 pg/mL). Through the elevation of glucagon level, subjects were more prone to be women, had higher platelet, total cholesterol, HDL-C and LDL-C (Table 1). Meanwhile, the prevalence of probable NASH showed a significantly increasing trend, but no significant trend was observed in NFS or prevalence of probable advanced fibrosis (Fig. 2).

Association of glucagon with NAFLD inflammatory progression in diabetic patients

Table 2 shows that glucagon was negatively associated with inflammatory progression of liver steatosis in diabetic patients. Although glucagon quartiles were not associated with simple NAFLD (*P* for trend = 0.176), they were negatively associated with probable NASH after adjustment for age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitors (Q4 vs. Q1 OR 0.71, 95% CI, 0.53–0.96, *P* for trend=0.010). Moreover, in ordinal regression, glucagon was also negatively associated with the inflammatory progression of NAFLD, with OR of 1SD increment of ln(glucagon) 0.77 (95% CI 0.64–0.91, *P* for trend=0.008).

Association of glucagon with advanced fibrosis in diabetic patients with NAFLD

Table 3 shows that glucagon was not significantly associated with fibrotic progression of liver steatosis in diabetic patients with NAFLD. Glucagon quartiles were not associated with indeterminate group (*P* for trend=0.298), nor were they were related to presence of advanced fibrosis (*P* for trend=0.751). The fibrotic progression of NAFLD was not associated with glucagon (*P* for trend=0.889).

Discussion

In this study including 4115 Chinese diabetic adults, we reported that glucagon quartiles were negatively

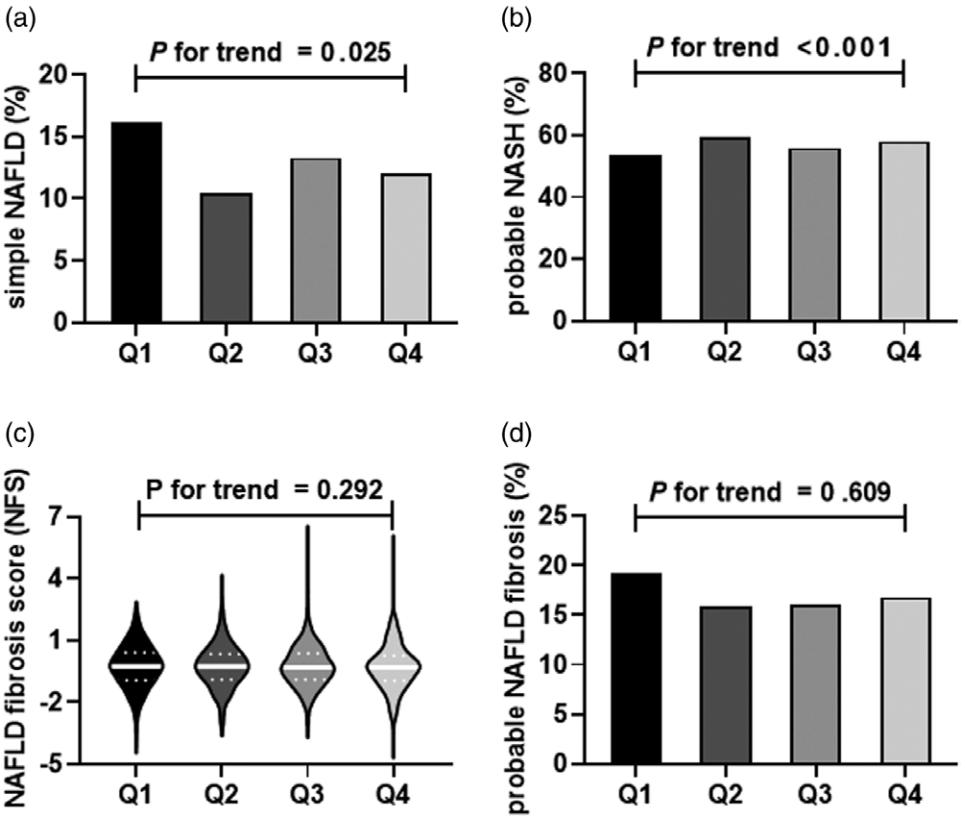

Fig. 2. Distribution of NAFLD inflammatory and fibrotic progression in different glucagon quartiles among Chinese diabetic patients.

Table 2. Relations of glucagon with NAFLD inflammatory progression in diabetic patients

	Glucagon, pg/mL					
	Quartile 1 (≤115.31)	Quartile 2 (115.32–154.12)	Quartile 3 (154.13–201.99)	Quartile 4 (>202)	*P* for trend	1SD increment of ln(glucagon)
Simple NAFLD	Reference	0.7 (0.49,1.00)	0.87 (0.62,1.22)	0.73 (0.51,1.03)	0.176	0.75 (0.58,0.97)
Probable NASH	Reference	0.91 (0.68,1.22)	0.75 (0.56,1.00)	0.71 (0.53,0.96)[a]	0.010	0.72 (0.58,0.90)[a]
Inflammatory progression of NAFLD	Reference	0.91 (0.72,1.15)	0.78 (0.62,0.99)[a]	0.75 (0.59,0.95)[a]	0.008	0.77 (0.64,0.91)[a]

Data are shown as regression odds ratios (95% CI). Multinomial and ordinal logistic regression analyses were used. Inflammatory progression of NAFLD: from non-NAFLD, simple NAFLD to probable NASH. The model was adjusted for age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitors.
[a]$P < 0.05$.

Table 3. Relations of glucagon with NAFLD fibrotic progression in diabetic patients

	Glucagon, pg/mL					
	Quartile 1 (≤115.31)	Quartile 2 (115.32–154.12)	Quartile 3 (154.13–201.99)	Quartile 4 (>202)	*P* for trend	1SD increment of ln(glucagon)
Indeterminate group	Reference	1.04 (0.69,1.57)	1.50 (0.97,2.31)	1.13 (0.76,1.70)	0.298	1.15 (0.87,1.51)
Presence of significant fibrosis	Reference	0.78 (0.46,1.33)	1.19 (0.69,2.06)	0.97 (0.57,1.64)	0.751	1.04 (0.72,1.50)
Fibrotic progression of NAFLD	Reference	0.87 (0.65,1.15)	1.03 (0.78,1.37)	0.96 (0.73,1.27)	0.889	1.00 (0.82,1.22)

Data are shown as regression odds ratios (95% CI). Multinomial and ordinal logistic regression analyses were used. Fibrotic progression of NAFLD: from absence of significant fibrosis, indeterminate results (NFS between −1.455 and 0.676) to presence of significant fibrosis (NFS >0.676). The model was adjusted for age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitors.

associated with probable NASH, independent of age, sex, duration of diabetes, current smoking, waist circumference, C-peptide, HbA1c, dyslipidemia, hypertension and use of incretins and SGLT2 inhibitors. Furthermore, glucagon was also negatively associated with the inflammatory progression of NAFLD from non-NAFLD, simple NAFLD to probable NASH. Whereas, glucagon was not significantly associated with fibrotic progression of liver steatosis in diabetic patients with NAFLD. Our results suggested that islet alpha cell function might influence the inflammatory progression of NAFLD in diabetic adults.

Several metabolic parameters in this study population were comparable among the subgroups divided by the quartiles of glucagon. The influence on glucagon from both T2DM and NAFLD could be the reason. Islet injury with alpha cell loss could reduce circulating glucagon, and

in some, but not all, individuals with T2DM increased concentrations of glucagon can be observed [20]. For fatty liver disease, the typical hormonal environment included hyperglucagonemia [21]. Hence, in patients with both T2DM and NAFLD, the glucagon level was not necessarily elevated. The comparable metabolic characteristics in the subgroups of glucagon quartiles could eliminate the effects of duration of diabetes, obesity (BMI and waist circumference), blood glucose (FPG and HbA1c), hypertension and dyslipidemia on the NAFLD progression.

Glucagon was inversely related to NAFLD progression both in this study and in other researches. The association between glucagon and NASH could be explained by the beneficial role of glucagon in NAFLD in both animal and human studies. Exogenous glucagon administration could reduce fatty liver in human and animal studies [22]. Glucagon had hypolipidemic effects on hepatocytes and promotes mobilization of hepatic fat [23]. In addition, attenuation of glucagon receptor signaling is proposed to be associated with an increased risk of fatty liver [24]. Furthermore, glucagon/T3 substantially improved hepatic fat content and improve NASH in preclinical disease models [25]. Therefore, it could be inferred that glucagon might play a protective role in the inflammatory progress of NAFLD in type 2 diabetic patients.

There are several potential mechanisms underlying the function of glucagon on the progression of NAFLD. Glucagon increases and insulin inhibits follistatin secretion both in vivo and in vitro, mediated via the secondary messenger cAMP in the hepatocyte. Circulating follistatin plays a negative role in regulating hepatic gluconeogenesis through β-cell. Follistatin reduces hepatic lipid uptake and synthesis suggesting that circulating follistatin could counteract the development of hepatic steatosis in NAFLD and NASH [26,27]. Moreover, PNPLA3, an early signature of carbohydrate-induced lipogenesis, was unchanged by addition of glucagon in HepG2 cells [28]. Besides, glucagon could induce acetylation of different energy-sensing factors, which was involved in the advancement of NAFLD to liver cancer [29].

The liver-alpha cell axis in T2DM patients has now been receiving great clinical interests [30]. Physiologically, glucagon controls amino acid metabolism in the liver, and the plasma concentration of amino acids regulates alpha cell secretion [30]. In steatosis liver, expression of several genes involved in amino acid uptake and degradation, and the glucagon receptor gene, were downregulated [31]. The concentration and function of glucagon were both altered and might lead to compensatory hyperglucagonemia, which was similar to the development process of insulin resistance. Therefore, the pathophysiological phenomenon that developed in parallel with hepatic steatosis could result in glucagon resistance. However, the role of glucagon in NAFLD inflammatory progression has not been fully elucidated. Given the close relationship between liver and alpha cells, further study about glucagon, NASH and the feedback loop involving amino acid in T2DM could contribute to the biology of glucagon in metabolic diseases.

In contrast to our previous findings that C-peptide was positively associated with inflammatory progression and negatively associated with fibrosis progression [12], in this study, we found that glucagon was negatively associated with inflammatory progression of fatty liver but did not show significant relation with fibrotic progression of NAFLD. The diverse association between C-peptide and NAFLD progression might be a result of different functions of pancreas alpha and beta cells. The pancreas alpha and beta cells control glucose metabolism by modulating the relative concentrations of glucagon and insulin. Unger suggested the 'bihormonal-abnormality' hypothesis in regards to the development of diabetes, stating that both relative and absolute hyperglucagonemia and insulin deficiency may be present in diabetic subjects [32]. As the overall islet cell dysfunction becomes worse as type 2 diabetes progresses, C-peptide to glucagon ratio exhibited a decreasing tendency (and glucagon-to-insulin ratio exhibited an increasing tendency) as the duration of diabetes became longer [33]. Moreover, the 'bihormonal-abnormality' (glucagon-to-insulin ratio) was suggested to be negatively correlated with ALT, suggesting the close relationship between the balance between β and α islet cells with liver diseases [33]. These results suggest that islet alpha- and beta-cell dysfunction might be closely related to the metabolic and inflammatory changes in NAFLD. Mechanically, the balance between β and α islet cells could regulate FGF-21 which were served as important endocrine metabolic regulators [34].

There are several limitations to this study. First, as a cross-sectional study, this study cannot identify a causal relationship between glucagon and NAFLD inflammatory progression. Second, NFS was used to assess probable advanced fibrosis in patients with NAFLD and type 2 diabetes. Liver histology is the gold standard to assess liver fibrotic stages, but this invasive examination could not be performed in large-scale epidemiology studies. NFS could accurately predict the presence or absence of advanced fibrosis in NAFLD, with high sensitivity and specificity, and was recommended to be used in clinical decisions to identify those who are suspicious for NAFLD and NASH in patients with type 2 diabetes [18]. Lastly, despite a large sample size, the study population was from a single center, so there could be selection bias.

In conclusion, we provide evidence for significant associations between glucagon and inflammatory progression of NAFLD, but not fibrotic progression of NAFLD. These results indicate that alpha cell function may have a role in the NAFLD inflammatory progression. Further unraveling the mechanism linking glucagon and NASH might provide new insight into the potential new drug targets.

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Y.L. and N.W. designed the study; Y.W., H.W., Z.L., W.Z., F.X., Y.C., X.C., C.W. and C.C. conducted the research; H.W., Y.W., Z.L. and N.W. analyzed the data and wrote the manuscript. The final manuscript was read and approved by all authors.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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