JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
ORIGINAL ARTICLE

Decreased plasma n6 : n3 polyunsaturated fatty acids ratio interacting with high C-peptide promotes non-alcoholic fatty liver disease in type 2 diabetes patients

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Keywords
Non-alcoholic fatty liver disease, Polyunsaturated fatty acids, Type 2 diabetes

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J Diabetes Investig 2021; 12: 1263–1271

doi: 10.1111/jdi.13469

ABSTRACT

Aims/Introduction: To explore relationships between polyunsaturated fatty acids (PUFA) and non-alcoholic fatty liver disease (NAFLD) in patients with type 2 diabetes, and whether insulin action has an interactive effect with PUFA on NAFLD progression.

Materials and Methods: We extracted clinical and omics data of 482 type 2 diabetes patients from a tertiary hospital consecutively from April 2018 to April 2019. NAFLD was estimated by ultrasound at admission. Plasma fasting n3 and n6 fatty acids were quantified by liquid chromatography–tandem mass spectrometry analysis. Restricted cubic spline nested in binary logistic regression was used to select the cut-off point, and estimate odds ratios and 95% confidence intervals. Additive interactions of the n6 : n3 ratio with insulin action for NAFLD were estimated using relative excess risk due to interaction, attributable proportion due to interaction and synergy index. Relative excess risk due to interaction >0, attributable proportion due to interaction >0 or synergy index >1 indicates biological interaction. Spearman correlation analysis was used to obtain partial correlation coefficients between PUFA and hallmarks of NAFLD.

Results: Of 482 patients, 313 were with and 169 were without NAFLD. N3 ≥800 and n6 PUFA ≥8,100 μmol/L were independently associated with increased NAFLD risk; n6 : n3 ratio ≤10 was associated with NAFLD (odds ratio 1.80, 95% confidence interval 1.20–2.71), and the effect size was amplified by high C-peptide (odds ratio 8.89, 95% confidence interval 4.48–17.7) with significant interaction. The additive interaction of the n6 : n3 ratio and fasting insulin was not significant.

Conclusion: Decreased n6 : n3 ratio was associated with increased NAFLD risk in type 2 diabetes patients, and the effect was only significant and amplified when there was the co-presence of high C-peptide.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by abnormal lipid accumulation, and can lead to a series of liver-related complications that affect life expectancy and quality[1]. Type 2 diabetes patients are at higher risk for NAFLD regardless of detection method, and >50% of them have NAFLD, as assessed by ultrasonography and proton magnetic resonance spectroscopy[2]. Notably, NAFLD and type 2 diabetes have bidirectional impacts on prognosis[3]. Patients with both type 2 diabetes and NAFLD have a higher risk of liver-specific complications, diabetes complications and mortality when compared with patients with either type 2 diabetes or NAFLD[4]. Considering these, the management of NAFLD in type 2 diabetes is of great clinical

Received 21 July 2020; revised 28 October 2020; accepted 12 November 2020

significance. Research into NAFLD in type 2 diabetes patients is urgently required[5].

Recently, research has shown that NAFLD is associated with a shift of lipid types and abundance, mostly with an increase in saturated fatty acids and monounsaturated fatty acids, and a decrease in polyunsaturated fatty acids (PUFAs)[6,7]. PUFAs include n3 fatty acids and n6 fatty acids. A decline in PUFA, especially n3 fatty acids, is frequently found in patients with cardiovascular diseases or NAFLD, leading to an increase in the n6 : n3 fatty acids ratio[8]. According to mechanism studies, n6 and n3 fatty acids are suggested to have some opposing impacts on cardiometabolic risk, with n3 fatty acids providing a protective effect, and n6 fatty acids having a harmful effect on health[9]. In this connection, plenty of randomized controlled trials found that supplement of n3 fatty acids in the form of diet or drugs can improve liver steatosis or liver function of patients with NAFLD[10]. Patients with diabetes are at high risk for NAFLD. Paradoxically, almost all these optimal findings were derived from patients without diabetes. Only one published study investigated the intervention in the setting of type 2 diabetes and reported an inconsistent finding. That randomized controlled trial found that the effect of PUFAs was inferior to a placebo in NAFLD patients with diabetes[11]. The role of PUFAs and their balance in the etiology and progression of hepatic steatosis in diabetes remain unclear.

Furthermore, impaired insulin sensitivity and subsequent compensatory insulin section were central components of type 2 diabetes, both of which might influence lipid ectopic deposition and promote hepatic steatosis[3,12]. In the meantime, fatty acids can induce abnormal insulin action through several pathways including oxidative stress, inflammation and so on[13]. Whether fatty acids and insulin action have an additive effect on NAFLD development in the context of diabetes is unknown.

Collectively, in the present hospital-based cross-sectional study, we aimed to investigate: (i) associations between PUFAs, especially their balance, that is, the n6 : n3 ratio, with NAFLD in type 2 diabetes patients; and (ii) the potential biological interaction of fatty acids and insulin action on the pathogenesis of NAFLD in type 2 diabetes patients.

METHODS

Study cohort

From April 2018 to April 2019, a total of 1,024 consecutive patients with type 2 diabetes were admitted into the Second Affiliated Hospital of Dalian Medical University (SAHDMU), Dalian, China, and agreed to participate in this research. Type 2 diabetes was diagnosed by the 1999 World Health Organization's criteria[14] or use of antidiabetic drugs; of them, 118 were excluded for secondary hepatic fat accumulation; 381 were further excluded for not having their plasma PUFAs measured on a voluntary basis at the patient's own expense; 43 were further excluded for not having an abdominal ultrasound scan. B-mode ultrasound was routinely recommended to inpatients with type 2 diabetes who could achieve it within 2 days from admission, except for patients who were not willing to pay for the fee (clinical and biochemical characteristics of participants according to conduction of B-mode ultrasound are described in Supporting Information). Finally, 482 patients were included in our primary analysis. The Ethics Committee for Clinical Research of SAHDMU approved the ethics of the study, and all the participants provided informed written consent.

Data collection and definitions

NAFLD was defined as having hepatic steatosis and no evidence for secondary hepatic fat accumulation. Secondary hepatic fat accumulation included significant alcohol consumption (ongoing alcohol consumption >21 drinks [~10 g of alcohol per one drink unit] on average per week for men, and >14 drinks on average per week for women), use of steatogenic medication or hereditary disorders. Hepatic steatosis was estimated by B-mode ultrasound according to liver echotexture: (i) mild steatosis, lightly thickened echo in the first half of the liver section; slight reduced echo in the second half of the liver; intrahepatic vessels absent; (ii) moderate steatosis, moderate thickened echo in the first half of the liver section; moderate reduced echo in the second half of the liver; intrahepatic vessels partial dimming; and (iii) severe steatosis, diffuse increase of echo in the first half of the liver section; heavily reduced echo in the second half of the liver; invisible intrahepatic vessels[15]. Liver stiffness measurement by vibration-controlled transient elastography was carried out for quantification of hepatic fibrosis. All inpatients were advised to have their fibrosis score measured if they were able to make an appointment during their hospitalization and were willing to pay the fee.

We also collected some essential and available clinical information. Age, sex, waist circumference, weight, height, duration of diabetes, systolic blood pressure, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glycated hemoglobin (HbA1c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting blood glucose, fasting insulin, C-peptide, diabetes medications, lipid-lowering drugs and diabetes complications were extracted based on an electronic medical system; AST ≥40 U/L and ALT ≥40 U/L were defined as abnormal liver enzymes; body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters.

Measurements of serum n3 and n6 PUFA

For patients included in the present study, fasting blood samples at admission were drawn and stored at −80°C for the following lipid profiles analysis.

Reagents: Water, acetonitrile and isopropyl alcohol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (>98%) and ammonium acetate (>99%) were obtained from Fluka (Buchs, Switzerland). Free fatty acids standards were obtained from Nu-Chek-Prep (Elysian, MN, USA).

Samples were thawed at 4°C. Formic acid and ammonium acetate plus water or acetonitrile and isopropyl alcohol were used as the mobile phase. C19:0 was used as the internal standard. Liquid chromatography–tandem mass spectrometry analysis was carried out with Eksigent LC100 and AB SCIEX Triple TOF 5600 (AB SCIEX, Framingham, MA, USA). Eksigent LC100 was equipped with XBridge Peptide BEH C18 Column (Waters, Milford, MA, USA). PeakView1.2 (AB SCIEX) was used for qualitative analysis. MultiQuant2.1 (AB SCIEX) was used for quantitative analysis.

Statistical analysis

Continuous variables are expressed as means (standard deviations) when normally distributed or medians (interquartile ranges) when skewed. Normality was checked by observing the Q-Q plot. Categorical variables are presented as frequencies (percentage). Differences between patients with NAFLD and without NAFLD were compared by the non-paired Student's *t*-test (or Mann–Whitney *U*-test when appropriate) for continuous variables, and the χ^2-test (or Fisher's test if appropriate) for categorical variables.

Binary logistic regression was carried out to estimate the odds ratios (ORs) and 95% confidence interval (CI). Restricted cubic spline nested in the logistic regression was used to examine potential non-linear relationships between n6, n3 fatty acids and their ratio with NAFLD risk, as before[14]. With restricted cubic spline, we identified the threshold where risk increased or decreased sharply, and then stratified PUFAs as categories according to these thresholds. Fatty acids were introduced into regression models as continuous variables (per standard deviation increase) and categories. We first carried out univariable analysis and then repeated analysis with adjustment for covariates, which included age, sex, HbA1c, systolic blood pressure, duration of diabetes, use of hypoglycemic drugs and lipid-lowering drugs. BMI, waist circumference, triglyceride, HDL-C, LDL-C, C-peptide, AST and ALT were not adjusted in the primary analysis considering that they might mediate associations between PUFAs and NAFLD or they were hallmarks of NAFLD. Instead, we adjusted for these variables in sensitive analysis. We also obtained correlation coefficients of the n6 : n3 ratio with these features of NAFLD, including BMI, waist circumference, HbA1c, FPG, triglyceride, HDL-C, LDL-C, C-peptide, liver fibrosis score, AST and ALT.

To estimate potential additive interactions of imbalanced PUFA with insulin action for NAFLD risk, we created four variables: (i) n6 : n3 ratio >10 and C-peptide <1.39 ng/mL (median, or fasting insulin <11.59 mU/L, as reference); (ii) n6 : n3 ratio >10 and C-peptide ≥1.39 ng/mL (or fasting insulin ≥11.59 mU/L); (iii) n6 : n3 ratio ≤10 and C-peptide <1.39 ng/mL (median, or fasting insulin <11.59 mU/L); (iv) n6 : n3 ratio >10 and C-peptide ≥1.39 ng/mL (median, or fasting insulin ≥11.59 mU/L). Relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP) and synergy index (S) were calculated to test the interactions. If any one of these three conditions; that is, RERI >0, AP >0 or S >1, was true, the biological interaction was statistically significant[16]. Age, sex, HbA1c, systolic blood pressure, duration of diabetes, hypoglycemic drugs and lipid-lowering drugs were adjusted in the primary analysis, whereas metabolic-associated parameters including BMI, waist circumference, HDL-C, LDL-C, triglyceride, AST and ALT were further adjusted in the sensitive analysis. To exclude bias from sex difference, we also carried out sex-matched analysis.

The most accurate method for the diagnosis of fatty liver was histology and proton magnetic resonance spectroscopy subsequently. Ultrasound-based measurements had lower sensitivity, but became the most popular screening method for their convenience and non-invasiveness. ALT had the worst sensitivity and specificity[5]. In the primary analysis, NAFLD and non-NAFLD were distinguished by ultrasound. In the sensitive analysis, we adjusted the definition; that is, patients with abnormal ultrasound imaging and ALT ≥40 U/L were assigned as NAFLD[17].

All analysis was carried out using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). *P*-values of <0.05 were considered statistically significant.

RESULTS

Characteristics of the study population

Of the 482 type 2 diabetes patients, 313 were with and 169 without NAFLD (Table 1). There were more women in the NAFLD group than in the non-NAFLD group. Patients with NAFLD had higher BMI, waist circumference, triglyceride, HbA1c, fasting insulin, homeostatic model assessment of insulin resistance, C-peptide, AST, ALT, n3 PUFA and n6 PUFA, and lower HDL-C and n6 : n3 ratio. In addition, compared with patients without NAFLD, those patients with NAFLD were younger (58.5, standard deviation 13.5 vs 62.5, standard deviation 11.7) and had shorter duration of diabetes. Systolic blood pressure, LDL-C, fasting blood glucose and liver fibrosis score were similar in the two groups (Table 1). Diabetic complications and medicine are shown in Table 1.

Associations between PUFA and NAFLD

As shown in Figure 1, n6 (Figure 1a) and n3 PUFA (Figure 1b) were positively associated with NAFLD non-linearly. N6 fatty acids ≥8,100 μmol/L increased 3.24-fold risk in univariate analysis (95% CI 1.60–6.55) and 2.69-fold risk in multivariate analysis (95% CI 1.27–5.67); n3 fatty acids ≥800 μmol/L increased 2.66-fold risk in univariate analysis (95% CI 1.59–4.43) and 2.20-fold risk in multivariate analysis (95% CI 1.28–3.78; Table 2). The n6 : n3 ratio was inversely associated with NAFLD non-linearly (Figure 1c). Compared with n6 : n3 ratio >10, n6 : n3 ratio ≤10 was associated with an increased risk of NAFLD in both the univariate (1.92, 95% CI 1.31–2.82) and multivariate model (1.80, 95% CI 1.20–2.71; Table 2). When further adjusted for variables-associated parameters, the association between the n6 : n3 ratio and NAFLD was still significant (Table S1).

Table 1 | Clinical and biochemical characteristics of participants according to the occurrence of non-alcoholic fatty liver disease

	NAFLD	Non-NAFLD	*P*-value
n	313	169	
Mild steatosis	274		
Moderate steatosis	33		
Severe steatosis	6		
Age (years)	58.5 ± 13.5	62.5 ± 11.7	0.0008
Duration of diabetes (years)	8 (2–15)	11 (6–20)	<0.0001
Male sex	125 (39.9)	86 (50.9)	0.0207
Waist circumference (cm)	94.7 ± 9.2	90.2 ± 9.3	<0.0001
Abnormal waist circumference	167 (53.4)	58 (34.3)	<0.0001
BMI (kg/m^2)	27.5 ± 3.6	25.1 ± 3.7	<0.0001
BMI <24.0 kg/m^2	52 (16.6)	52 (16.6)	<0.0001
BMI ≥24 and <28.0 kg/m^2	130 (41.5)	78 (46.2)	
BMI ≥28.0 kg/m^2	131 (41.9)	31 (18.3)	
Systolic blood pressure (mmHg)	150.4 ± 21.3	148.3 ± 21.4	0.2949
HDL-C (mmol/L)	1.19 ± 0.30	1.29 ± 0.33	0.0008
LDL-C (mmol/L)	2.57 ± 0.82	2.52 ± 0.85	0.5843
Triglyceride (mmol/L)	1.73 (1.30–2.66)	1.23 (0.87–1.67)	<0.0001
HbA1c (%)	8.50 (7.30–10.10)	8.00 (6.80–9.40)	0.0009
Fasting blood glucose (mmol/L)	9.88 ± 3.56	9.04 ± 3.59	0.5843
C-peptide (ng/mL)	1.60 (1.10–2.17)	1.03 (0.74–1.44)	<0.0001
Fasting insulin (mU/L)	13.1 (8.5–21.5)	9.5 (5.7–18.7)	0.0001
Liver fibrosis score (kPa)	7.1 (6.0–8.9)	7.0 (5.7–9.0)	0.4975
AST (U/L)	20.28 (16.42–26.15)	18.10 (15.50–23.26)	0.0022
AST ≥40 U/L	29 (9.3)	4 (2.4)	0.0039
ALT (U/L)	23.99 (16.53–36.35)	18.55 (13.75–25.17)	<0.0001
ALT ≥40 U/L	60 (19.2)	10 (5.9)	<0.0001
n3 PUFA (μmol/L)	5,830.97 (4,832.26–7,241.60)	5,355.43 (4,518.18–6,457.55)	0.0022
n3 PUFA ≥800 μmol/L	53 (16.9)	10 (5.9)	0.0006
n6 PUFA (μmol/L)	592.47 (450.84–834.55)	475.45 (383.72–623.56)	<0.0001
n6 PUFA ≥8,100 μmol/L	89 (28.4)	22 (13.0)	0.0001
n6/n3 ratio	10.3 ± 3.9	11.7 ± 4.1	0.0002
n6/n3 ratio ≤10	167 (53.4)	63 (37.3)	0.0007
Prior CAD	32 (10.2)	13 (7.7)	0.3620
Prior stroke	13 (4.2)	11 (6.5)	0.2767
Diabetic retinopathy	79 (26.3)	70 (42.4)	0.0003
Diabetic nephropathy	144 (46.0)	80 (47.3)	0.7798
Hypoglycemic drugs	293 (93.6)	166 (98.2)	0.0245
Insulin (drug)	175 (55.9)	108 (63.9)	0.0889
Metformin	164 (53.3)	44 (26.2)	<0.0001
Thiazolidinedione	57 (18.5)	6 (3.6)	<0.0001
Lipid-lowering drugs	162 (51.8)	66 (39.1)	0.0077

Data are the mean ± standard deviation, median (interquartile range) or *n* (%). *P*-values were derived from independent-samples Student's *t*-test for normally distributed variables, Mann–Whitney *U*-test for skewed distributions and χ^2-test (or Fisher's test if appropriate) for categorical variables. Abnormal waist circumference, men ≥102 cm or women ≥88 cm. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; PUFA, polyunsaturated fatty acids.

In addition, a higher n6 : n3 ratio was inversely correlated with triglyceride (correlation coefficient −0.10, $P = 0.0364$) and positively correlated with HbA1c (correlation coefficient 0.10, $P = 0.0364$) weakly after adjusted for traditional risk factors. Other hallmarks were not significantly correlated with the n6 : n3 ratio (Table 3).

Additive interaction of the n6 : n3 ratio with insulin action for NAFLD

Using a high n6 : n3 ratio and low C-peptide as reference, the OR of C-peptide >1.39 only was 3.13 (95% CI 1.77–5.55), and OR of n6 : n3 ratio ≤10 only was 1.40 (95% CI 0.80–2.42); the co-presence of a low n6 : n3 ratio and high C-peptide greatly

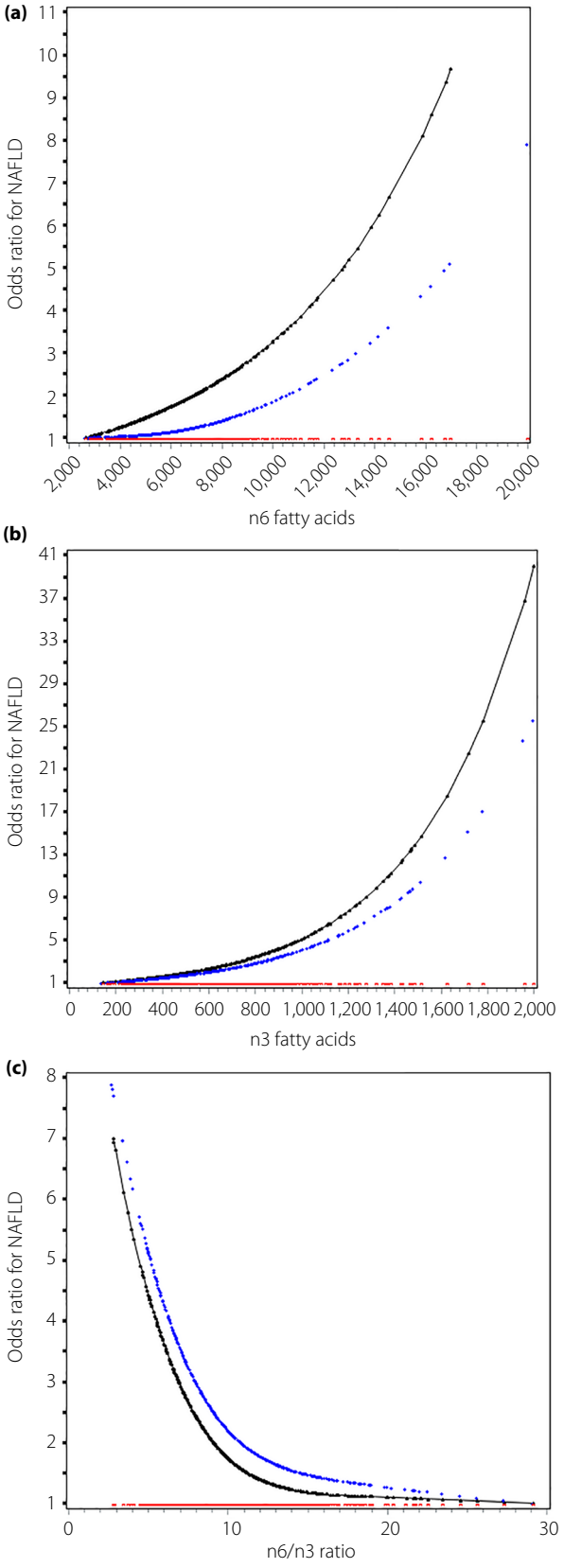

Figure 1 | Odds ratio curves of polyunsaturated fatty acids for non-alcoholic fatty liver disease (NAFLD) risk in type 2 diabetes patients. (a–c) The relationships between n6, n3 fatty acids and the n6 : n3 ratio with NAFLD risk, respectively. The black curve was derived from univariable analysis, and the blue curve derived from multivariable analysis that adjusted for age, sex, glycated hemoglobin, systolic blood pressure, duration of diabetes, hypoglycemic drugs and lipid-lowering drugs. The red curve represents the reference level (i.e., the odds ratio for type 2 diabetes mellitus was 1).

increased the OR to 8.89 (95% CI 4.48–17.7). The additive interaction measures were significant (RERI 5.37, 95% CI −0.12 to 10.9; AP 0.60, 95% CI 0.33–0.88; S 3.12, 95% CI 1.29–7.54; Table 4).

With a high n6 : n3 ratio and low fasting insulin as the reference, the OR of fasting insulin >11.59 mU/L only was 2.53 (95% CI 1.45–4.41), and the OR of a n6 : n3 ratio ≤10 only was 2.58 (95% CI 1.47–4.53); the OR of co-presence of a low n6 : n3 ratio and high fasting insulin was 3.20 (95% CI 1.75–5.85). The additive interaction was not significant (RERI −0.91, 95% CI −3.09 to 1.28; AP −0.28, 95% CI −1.03 to 0.47; S 0.71, 95% CI 0.31–1.60; Table 4).

In addition, these findings were robust when further adjusted for metabolic-associated parameters (Table S2) or in sex-matched analysis (Table S3). When changing the definition of NAFLD to the co-presence of abnormal abdominal ultrasonography and AST ≥40 U/L, there were 253 and 229 patients with and without NAFLD, respectively. The associations between the n6 : n3 ratio and NAFLD were slightly attenuated. but remained significant (1.59 95% CI 1.10–2.31). The interaction between a low n6 : n3 ratio and high C-peptide still existed (RERI 1.88, 95% CI 0.23–3.53; AP 0.53, 95% CI 0.24–0.83; S 0.48, 95% CI 0.82–17.9), whereas the interaction between a low n6 : n3 ratio and high fasting insulin was still non-significant (RERI −1.17, 95% CI −2.66 to 0.33; AP −0.65, 95% CI −1.55 to 0.24; S 0.40, 95% CI 0.14–1.15; Table S4).

Among 525 patients who had their lipids measured, compared with participants who did not have B-mode ultrasound, participants who had B-mode ultrasound were older and had higher systolic blood pressure, fasting blood glucose and lower fasting insulin. Other characteristics were the same between the two groups (Table S5).

DISCUSSION

In the present study, we found that in the type 2 diabetes population, the presence of NAFLD was associated with higher n6 and n3 PUFA levels, but a lower n6 : n3 ratio. In addition, we detected an interaction between a low n6 : n3 ratio and high C-peptide for NAFLD risk. The inverse association between the n6 : n3 ratio and NAFLD risk was only observed in patients with higher C-peptide, suggesting that increased risk of NAFLD in

Table 2 | Relationships between polyunsaturated fatty acids and non-alcoholic fatty liver disease in patients with type 2 diabetes

	Univariable model		Multivariable model	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
n6 PUFA				
Per SD increase	1.43 (1.14–1.79)	0.0019	1.24 (0.97–1.58)	0.0864
≥8,100 vs <8,100 μmol/L	3.24 (1.60–6.55)	0.0011	2.69 (1.27–5.67)	0.0096
n3 PUFA				
Per SD increase	1.77 (1.34–2.35)	<0.0001	1.94 (1.49–2.52)	<0.0001
≥800 vs <800 μmol/L	2.66 (1.59–4.43)	0.0002	2.20 (1.28–3.78)	0.0045
n6/n3 ratio				
Per SD increase	0.71 (0.58–0.85)	0.0003	0.69 (0.56–0.86)	0.0007
≤10 vs >10	1.92 (1.31–2.82)	0.0008	1.80 (1.20–2.71)	0.0045

Multivariable model was adjusted for age, sex, glycated hemoglobin, systolic blood pressure, duration of diabetes, hypoglycemic drugs and lipid-lowering drugs. CI, confidence interval; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; PUFA, polyunsaturated fatty acids; SD, standard deviation.

Table 3 | Spearman correlation of the n6 : n3 ratio with features of non-alcoholic fatty liver disease

	Correlation coefficients	*P*-value
Univariable analysis		
BMI	−0.08	0.0863
Waist circumference (cm)	−0.05	0.2421
Fasting blood glucose (mmol/L)	0.04	0.4300
HbA1c (%)	0.07	0.1287
HDL-C (mmol/L)	−0.09	0.0491
LDL-C (mmol/L)	0.02	0.7117
Triglyceride (mmol/L)	−0.13	0.0050
C-peptide (ng/mL)	−0.08	0.0627
Liver fibrosis score (kPa)	−0.05	0.3547
AST (U/L)	−0.02	0.6838
ALT (U/L)	−0.06	0.1774
Multivariable analysis		
BMI	−0.06	0.1798
Waist circumference (cm)	−0.04	0.4003
Fasting blood glucose (mmol/L)	0.05	0.2451
HbA1c (%)	0.11	0.0364
HDL-C (mmol/L)	−0.08	0.0785
LDL-C (mmol/L)	0.04	0.3913
Triglyceride (mmol/L)	−0.10	0.0342
C-peptide (ng/mL)	−0.07	0.1247
Liver fibrosis score (kPa)	−0.04	0.4615
AST (U/L)	−0.03	0.5155
ALT (U/L)	−0.09	0.0669

Multivariable model was adjusted for age, gender, systolic blood pressure, duration of diabetes, hypoglycemic drugs and lipid-lowering drugs. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease.

type 2 diabetes with a low n6 : n3 ratio was conditional on the presence of high C-peptide. However, we did not find a significant interaction between the n6 : n3 ratio and fasting insulin.

Normally, most fatty acids are stored in adipose tissues. In insulin-resistant states, lipolysis of adipose tissues accelerated and circulating free fatty acids were increased[18]. Despite many studies finding a decline in hepatic PUFA content, it was still rational that we found circulating PUFA levels elevated in insulin-resistant states[6,19]. The presence of NAFLD in type 2 diabetes was associated with more severe insulin resistance and hyperinsulinemia, whereas impaired insulin signaling mediated exacerbated lipid mobilization from adipose tissues to the liver in turn[20,21]. Also, patients with NAFLD in the present study had higher BMIs, which provided a larger lipid pool. Therefore, the present results suggested that higher plasma free n3 and n6 PUFA were potential markers of aggravating β-cell function and obesity in type 2 diabetes patients with NAFLD.

Apart from absolute levels, the balance of n6 and n3 PUFA was very important. A great deal of evidence showed that n3 performed better than n6 fatty acids in weight loss and inflammation[9,22]. In this connection, experiments and meta-analysis had encouraged the use of n3 PUFAs as specific treatment options for NAFLD progression[23,24]. However, in the present study, a higher n6 : n3 ratio was associated with a lower NAFLD risk in diabetes patients. One possibility is that n3 PUFA provides insufficient protection from other fatty acids and is overconsumption, which contributes to a higher n6 : n3 ratio and lower NAFLD risk. Nevertheless, clinical studies showed that n3 PUFA supplement did not perform well in diabetes patients[11,25], whereas n6 PUFA reduced the incidence of type 2 diabetes[26,27]. Monaco *et al.*[28] reported that in obese Zucker rats, consumption of α-linolenic acid attenuated insulin signaling, weakened mitochondrial respiration and increased formation of reactive oxygen species (ROS); despite benign performance in rats without severe metabolic disturbance[29], some other studies showed that in rats with both severe insulin resistance and hyperinsulinemia, n3 PUFA reversed the compensatory increase in insulin secretion, but did not improve adequate insulin sensitivity, leading to poorer metabolism[30,31]. In this regard, the second possibility is that a low n6 : n3 ratio

Table 4 | Additive interaction of the n6 : n3 ratio with insulin action for non-alcoholic fatty liver disease in patients with type 2 diabetes

	Univariable model		Multivariable model	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
C-peptide ≥ vs <1.39 ng/mL	4.05 (2.70–6.06)	<0.0001	4.13 (2.66–6.43)	<0.0001
Fasting insulin ≥ vs <11.59 mU/L	1.85 (1.27–2.71)	0.0015	1.77 (1.17–2.67)	0.0067
Interaction of n6 : n3 ratio with insulin secretion				
n6 : n3 ratio >10 & C-peptide <1.39 ng/mL	Reference		Reference	
n6/n3 ratio >10 & C-peptide ≥1.39 ng/mL	3.12 (1.85–5.28)	<0.0001	3.13 (1.77–5.55)	<0.0001
n6 : n3 ratio ≤10 & C-peptide <1.39 ng/mL	1.50 (0.90–2.51)	0.1217	1.40 (0.80–2.42)	0.2367
n6 : n3 ratio ≤10 & C-peptide ≥1.39 ng/mL	8.79 (4.63–16.7)	<0.0001	8.89 (4.48–17.7)	<0.0001
Measure				
RERI	5.16 (0.02–10.3)		5.37 (–0.12–10.9)	
AP	0.59 (0.31–0.86)		0.60 (0.33–0.88)	
S	2.97 (1.29–6.81)		3.12 (1.29–7.54)	
Interaction of n6 : n3 ratio with fasting insulin				
n6 : n3 ratio >10 & insulin <11.59 mU/L	Reference		Reference	
n6 : n3 ratio >10 & insulin ≥11.59 mU/L	2.74 (1.63–4.60)	0.0001	2.53 (1.45–4.41)	0.0011
n6 : n3 ratio ≤10 & insulin <11.59 mU/L	2.88 (1.69–4.89)	<0.0001	2.58 (1.47–4.53)	0.0009
n6 : n3 ratio ≤10 & insulin ≥11.59 mU/L	3.61 (2.05–6.36)	<0.0001	3.20 (1.75–5.85)	0.0002
Measure				
RERI	–1.00 (–3.30–1.30)		–0.91 (–3.09–1.28)	
AP	–0.28 (–0.98–0.43)		–0.28 (–1.03–0.47)	
S	0.72 (0.35–1.51)		0.71 (0.31–1.60)	

Low and high level of C-peptide and fasting insulin was defined according to less or more than medians. Multivariable model was adjusted for age, sex, glycated hemoglobin, systolic blood pressure, duration of diabetes, hypoglycemic drugs and lipid-lowering drugs. Significant elative excess risk due to interaction (RERI) >0, attributable proportion due to interaction (AP) >0 or synergy index (S) >1 indicates a significant additive interaction. CI, confidence interval; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

was derived from relative decreased n6 and increased n3 fatty acids; that is, higher n3 PUFA led to increased NAFLD risk. Whether NAFLD together with type 2 diabetes changed the response of n3 PUFA for hepatic steatosis is unknown. Indeed, it is worthwhile to investigate the role of the balance of n6 and n3 PUFA for NAFLD in diabetes patients. It was important to note that n6 : n3 is positively correlated with HbA1c, which is the opposite to the effect of n6 : n3 on NAFLD. It is possible that an unbalanced n6 : n3 ratio can raise postprandial blood glucose through a pathway other than liver insulin resistance; for example, decreasing capacity of glucose conversion to fatty acids through carbohydrate regulatory element-binding protein and Max-like factor-X[32].

C-peptide is a byproduct of proinsulin, and releases from β-cells in equimolar amounts with insulin[33]. Both C-peptide and insulin were identified as important predictors and markers of conditions, such as insulin resistance, diabetes, NAFLD and cardiovascular diseases[34,35]. Compared with plasma insulin, C-peptide is almost unaffected by the first metabolism of the liver and has a much longer half-life[33]. Therefore, C-peptide levels can always represent endogenous insulin secretion more appropriately than insulin levels, and has better performance than insulin in predicting NAFLD, insulin resistance and type 2 diabetes[34–36]. In the present study, we found that the adverse effect of a decreased n6 : n3 ratio depended on the presence of a high level of C-peptide. As speculated before, if n3 PUFA was protective and overconsumed, then in patients with severe insulin resistance, n3 PUFA cannot appropriately protect patients from fatty liver; if n3 PUFA was harmful in type 2 diabetes, then the accumulation of n3 PUFA interacted with insulin resistance for fatty liver. Insulin resistance might modify the effect of PUFA[37,38]. Furthermore, recent evidence supported that C-peptide was not only a biomarker of insulin action, but also of biological effects, such as pro-inflammation in the setting of insulin resistance, which might also interplay with PUFA for hepatic steatosis[39].

The present findings had important clinical and mechanistic implications. Diabetes patients with NAFLD are susceptible to unfavorable prognosis. However, related diagnosis and treatment are optimal. PUFA emerges as a new option. Our study found an inverse relationship between the n6 : n3 ratio and NAFLD development in type 2 diabetes, which is opposite to findings in the population without type 2 diabetes. Thus, the present study not only generated some new hypotheses for basic scientists to investigate the molecular mechanisms underlying liver fat accumulation in diabetes, but also suggested the balance of PUFA as a potential non-invasive marker of diagnosis and therapeutic target. In addition, as the association between PUFA and NAFLD relies on C-peptide, maybe combination medication is likely to offer an additive benefit.

The present study also had several limitations. First, our research was retrospective, so whether a reduced n6 : n3 ratio is a cause or consequence of NAFLD requires more prospective

studies. Second, we did not collect information on physical activity and diet. Nevertheless, physical activity and diet were associated with BMI and waist circumference. We adjusted BMI and waist circumference in sensitive analysis, and the association between the n6 : n3 ratio and NAFLD was still significant. Third, although compared with confirmatory diagnosis (liver biopsy or proton magnetic resonance spectroscopy), diagnosis can be missed by ultrasonography, the resolution of fatty liver, as assessed by ultrasonography, has been found to reduce the risk of type 2 diabetes development to a level similar to individuals without NAFLD[40], so liver steatosis assessed by ultrasonography can be of significant clinical implication. Fourth, most of our participants had mild hepatic steatosis. Therefore, it was plausible that the fibrosis score was similar between participants with and without NAFLD, and no significant correction was found between the n6 : n3 ratio and fibrosis score. In the future, more patients with moderate and severe steatosis should be included. Fifth, patients who had an abdominal ultrasound had more unfavorable metabolism, but a similar n6 : n3 ratio compared with their counterparts who did not have an abdominal ultrasound. Therefore, there might be more NAFLD in the latter group, and we might overestimate the effect size of the n6 : n3 ratio for NAFLD.

In conclusion, we found that a decreased n6 : n3 ratio increased the NAFLD risk in type 2 diabetes patients, and the coexistence of high C-peptide further amplified the effect size. More prospective studies and basic science are warranted to elaborate the role of PUFAs for liver fat accumulation in the context of type 2 diabetes.

ACKNOWLEDGMENTS

The authors thank all the physicians and doctors at The Second Affiliated Hospital of Dalian Medical University who participated in the study and extended their support for data collection. The authors also thank all the laboratory technicians at Dalian Institute of Chemical Physics and RSKT Biopharma Inc, Dalian, Liaoning, for measuring the metabolites. This work was supported by the project for the National Key Research and Development Program of China (2019YFA0802302, 2019YFA0802300), State Key Project on Infectious Diseases of China (2018ZX10723204).

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Relationships between the n6 : n3 ratio and non-alcoholic fatty liver disease in patients with type 2 diabetes.

Table S2 | Interactions between the n6 : n3 ratio and insulin action for non-alcoholic fatty liver disease risk with further adjustment of metabolic-associated parameters.

Table S3 | Sex-matched analysis* for associations between the n6 : n3 ratio and non-alcoholic fatty liver disease ,and its interaction with insulin action.

Table S4 | Association of the n6 : n3 ratio and non-alcoholic fatty liver disease[$] and its interaction with insulin action.

Table S5 | Clinical and biochemical characteristics of participants according a B-mode ultrasound.