

## Original Article

# Plasma phospholipid polyunsaturated fatty acids and homocysteine in Chinese type 2 diabetes patients

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The main aim of the present study was to investigate the plasma phospholipids (PL) fatty acids status and its association with plasma Hcy in patients with type 2 diabetes mellitus (T2DM). One hundred and four T2DM (aged 57.3±13.4 y) and 150 healthy subjects (aged 48.4±8.7 y) were recruited. Plasma Hcy and PL fatty acids were determined by standard methods. Plasma Hcy concentration in T2DM was significantly higher than that in healthy subjects ( $p<0.001$ ). The prevalence of hyperhomocysteinemia was significantly higher in T2DM (36.54%) than that in healthy subjects (17.32%) ( $p=0.012$ ). Plasma PL 20:4n-6 ( $r=0.303$ ,  $p=0.012$ ), 22:5n-3 ( $r=0.312$ ,  $p=0.01$ ), total PUFA ( $r=0.303$ ,  $p=0.012$ ), n-6 PUFA ( $r=0.261$ ,  $p=0.032$ ) were significantly positively associated with plasma Hcy concentration in T2DM. While, plasma PL n-3:n-6 PUFA ( $r=-0.400$ ,  $p=0.046$ ) was negatively associated with plasma Hcy in T2DM. In healthy subjects, plasma PL 22:6n-3 ( $r=-0.201$ ,  $p=0.042$ ) was negatively associated with plasma Hcy. In addition, plasma PL 22:6n-3 ( $r=0.193$ ,  $p=0.044$ ) and 22:5n-6 ( $r=0.234$ ,  $p=0.038$ ) were significantly negatively associated with plasma vitamin B-12 in healthy subjects. Our results suggested that increased plasma Hcy levels in T2DM associated with low n-3:n-6 ratio intake. We suggest that T2DM increase their long chain n-3 PUFA intake from fish or fish oil while decrease n-6 PUFA intake.

**Key Words:** n-3 polyunsaturated fatty acid, homocysteine, type 2 diabetes, phospholipids, monounsaturated fatty acids

## INTRODUCTION

Diabetes mellitus is one of the most common chronic diseases in nearly all countries, and continues to increase in numbers and significance. Its rapidly increasing global prevalence is a primary cause of concern. The diabetes epidemic is fueled by a societal increase in insulin resistance, caused by lifestyle factors, particularly excessive caloric intake and physical inactivity.<sup>1</sup> It is estimated that in 2030 around 366 million people could be suffering from diabetes among the adult population of the world.<sup>2</sup> Therefore, diabetes is regarded as a serious condition that affects both individuals and society as a whole.

Pathogenesis of type 2 diabetes mellitus (T2DM) has been extensively investigated. Several mechanisms have been proposed, including increased non-esterified fatty acids, inflammatory cytokines, adipokines, and mitochondrial dysfunction for insulin resistance, and glucotoxicity, lipotoxicity, and amyloid formation for  $\beta$ -cell dysfunction. Moreover, elevated blood homocysteine (Hcy), a risk factor in cardiovascular diseases,<sup>3</sup> is also associated with coronary and peripheral vascular diseases.<sup>4,5</sup> In patients with diabetes, elevated Hcy levels have been reported to be associated with endothelial dysfunction, insulin resistance, prothrombotic state, macroangiopathy and nephropathy.<sup>6</sup> However, the results on the association between plasma Hcy levels and T2DM were conflicting.<sup>7-9</sup>

Daily intake of n-3 polyunsaturated fatty acids (PUFA)

has a positive effect on metabolic diseases such as, cardiovascular disease (CVD),<sup>10-11</sup> T2DM,<sup>12</sup> and the metabolic syndrome.<sup>13</sup> Furthermore, platelet/plasma PL n-3 PUFA was negatively associated with plasma Hcy in middle aged and geriatric hyperlipaemia patients and in healthy male subjects.<sup>14-15</sup> The intervention study demonstrated that consumption of n-3 PUFA supplements (3 g/day) for 2 months decreases the levels of Hcy in T2DM.<sup>16</sup> Animal study and human studies have elucidated the potential mechanism by which n-3 PUFA regulate Hcy metabolism.<sup>17-19</sup>

However, the association between plasma Hcy concentration and plasma PL fatty acids has not been investigated in Chinese T2DM. The association of plasma Hcy with T2DM is still a matter of debate. The present study was carried out in order to determine plasma Hcy concentration in T2DM and its association of plasma PL fatty acids in Chinese T2DM.

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**Table 1.** The demographic and biochemical measurements in type 2 diabetes and healthy subjects

	T2DM	Healthy subjects	<i>p</i> -value
	(n=104)	(n=150)	
Age, year	57.3 ± 13.4	48.4 ± 8.7	0.021
BMI, kg/m <sup>2</sup>	24.6 ± 2.4	24.3 ± 2.7	0.432
SBP, mmHg	138 ± 24.1	126 ± 17.4	<0.001
DBP, mmHg	81.0 ± 12.1	76.9 ± 13.1	0.018
Glucose, mmol/L	11.3 ± 4.56	5.35 ± 0.89	<0.001
Insulin, uIU/mL	6.47 ± 3.19	5.52 ± 5.18	<0.001
TC, mmol/L	4.96 ± 1.29	5.54 ± 0.97	<0.001
LDL-C, mmol/L	3.02 ± 1.00	2.71 ± 0.69	0.015
HDL-C, mmol/L	1.37 ± 0.33	1.29 ± 0.30	0.090
TG, mmol/L	1.89 ± 0.98	1.98 ± 1.09	0.583
Plasma Hcy, µmol/L	14.0 ± 4.52	10.6 ± 3.63	<0.001
Plasma LogVitamin B <sub>12</sub>	6.46 ± 0.61	5.87 ± 0.36	<0.001
Plasma Folate, ng/mL	8.37 ± 3.16	7.07 ± 3.68	0.007

Data was expressed as Mean ± SD.

*p*<0.05 was considered as significant differences.

TC: total cholesterol, TG: Total triglyceride, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure,

## METHODS AND MATERIALS

### Subjects

The study protocol was approved by the Ethics Committee, College of Biosystem Engineering and Food Science, Zhejiang University, China, and all subjects were volunteers who gave their written consent prior to participation in the study.

Type 2 diabetes was defined as meeting one or more of the following criteria: (1) fasting glucose  $\geq 7.0$  mmol/l; (2) 2-h glucose  $\geq 11.1$  mmol/l; or (3) previous diagnosis of type 2 diabetes.<sup>20</sup> After screening, one hundred and four T2DM (aged 57.3±13.4 y) were recruited from outpatients in the Shaoxing Hospital, Shaoxing, China. Blood sugar levels were stabilized between 7.0-10.0 mmol/L. Subjects with type 1 DM, patients with a history of cardiovascular disease and/or arteriosclerotic disease, cerebrovascular disease, serious hepatic disease and/or renal disease and haematological disorders were excluded from the study.

Healthy subjects were recruited through a health check program during the period of October 2010 through March 2011 in the Zhejiang Hospital. After screening for hypertension, renal disease, hyperlipaemia, haematological disorders, diabetes, and family history of cardiovascular disease, excessive alcohol intake and drug use, 150 subjects (aged 48.4±8.7 y) were included.

### Blood collection

Subjects attended the Hospital in the morning following an overnight fast. Subjects were allowed to sit relaxed for 10 min, the subject's weight, height, waist to hip ratio and blood pressure were measured. Then venous blood was taken in plain and EDTA vacuum tubes with 21-gauge needles. After blood collection, plasma samples were prepared quickly after blood was drawn, aliquoted into separate tubes and stored at -20°C until analysis.

### Laboratory measurements

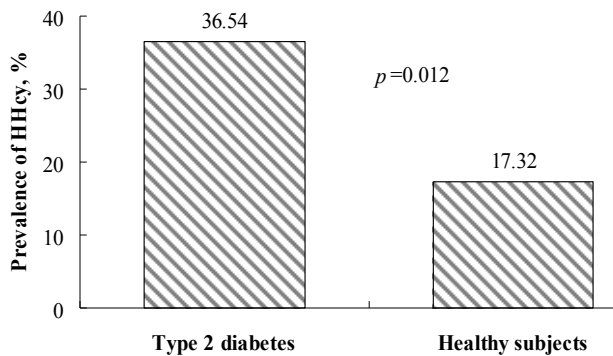
Plasma total Hcy was determined by polarized fluorescence immunoassay in an AXSYM system.<sup>21</sup> Plasma lipids were determined on an autoanalyser (Olympus AU2700, Tokyo, Japan), via commercially available kits (Olympus, Tokyo, Japan). Fasting glucose were measured by standard methods as previously described.<sup>22</sup> Total lipid content of plasma was extracted with solvents, the PL fraction was separated by thin layer chromatography (TLC) and the fatty acid methyl esters were prepared and separated by gas-liquid chromatography as described previously.<sup>23</sup>

### Statistical analysis

Data analyses were performed using SAS for Windows, version 9.1 (SAS Institute). All continuous variables were examined for normal distribution. Differences between the two groups for each outcome were analyzed using two-sample *t* test. The associations between plasma PL fatty acid composition and Hcy were determined by partial correlation, controlling for potential confounding factors. A Mantel-Haenszel chi-square test was used to calculate the *p*-value and the prevalence of hyperhomocysteinemia (HHcy) in T2DM and healthy subjects. A multi-variable stepwise regression model adjusted for age, gender, BMI, vitamin B-12, folate, Glucose, HDL-C, LDL-C, TC, TG, and insulin was created to identify potential independent correlates of plasma Hcy. All data are expressed as mean ± SD. Differences between groups were considered to be statistically significant at *p*<0.05.

## RESULTS

Systolic blood pressure (*p*<0.001), diastolic blood pressure (*p*=0.018), fasting glucose (*p*<0.001), insulin (*p*<0.001), low density lipoprotein cholesterol (LDL-C) (*p*=0.015) in T2DM was significantly higher than in healthy subjects. While, total triglyceride (TG), high density lipoprotein cholesterol (HDL-C) was not significantly different between these two groups (Table 1).



**Figure 1.** The prevalence of HHcy in type 2 diabetes and healthy subjects. A Mantel-Haenszel chi-square test was used to calculate the  $p$ -value.

**Table 2.** Independent predictors of plasma Hcy after adjustment for other clinical characteristics and risk factors

Predictors ( $R^2=0.30$ )	$\beta$ Coefficient	$p$
Age	0.193	<0.001
Vitamin B-12	-0.240	0.001
Folate	-0.373	0.015

Plasma Hcy concentration in T2DM was significantly higher than in healthy subjects ( $p<0.001$ ). The prevalence of HHcy was significantly higher in T2DM (36.54%) than in healthy subjects (17.32) (Figure 1).

Multivariable regression analysis that adjusted for age, gender, BMI, vitamin B-12, folate, Glucose, HDL-C, LDL-C, TC, TG, and Insulin was created to identify potential independent correlates of plasma Hcy. The only significant correlates of Hcy were age ( $p<0.001$ ), vitamin B-12 ( $p=0.001$ ), and folate ( $p=0.015$ ). These variables explained 30% of the variance in plasma Hcy (Table 2). Plasma PL 16:0 ( $r=0.269$ ,  $p=0.026$ ), 18:1n-9 ( $r=0.246$ ,  $p=0.043$ ), 18:1n-7 ( $r=0.288$ ,  $p=0.017$ ), 20:4n-6 ( $r=0.303$ ,  $p=0.012$ ), 22:5n-3 ( $r=0.312$ ,  $p=0.010$ ), total PUFA ( $r=0.303$ ,  $p=0.012$ ), n-6 PUFA ( $r=0.261$ ,  $p=0.032$ ) were significantly positively associated with plasma Hcy concentration. While, plasma PL n-3:n-6 PUFA ( $r=-0.400$ ,  $p=0.046$ ) was negatively associated with plasma Hcy in T2DM (Table 3).

Plasma PL 18:1n-9 ( $r=0.210$ ,  $p=0.041$ ) was significantly positively associated with plasma Hcy concentration. While, plasma PL 22:6n-3 ( $r=-0.201$ ,  $p=0.042$ ) was negatively associated with plasma Hcy in healthy subjects (Table 3). In addition, plasma PL 22:6n-3 ( $r=0.193$ ,  $p=0.044$ ) and 22:5n-6 ( $r=0.234$ ,  $p=0.038$ ) were significantly negatively associated with plasma vitamin B-12.

## DISCUSSION

In the present study, we mainly found that plasma Hcy concentration in T2DM was significantly higher than in healthy subjects. Plasma 20:4n-6, total PUFA, n-6 PUFA were significantly positively associated with plasma Hcy concentration. While, plasma PL n-3:n-6 PUFA was negatively associated with plasma Hcy in T2DM. Plasma PL 22:6n-3 was negatively associated with plasma Hcy in healthy subjects.

Hcy is an independent cardiovascular risk factor.<sup>24-25</sup> An association between elevated levels of Hcy and the vascular complications of diabetes has also been reported.<sup>6</sup> While, studies on circulating Hcy levels in T2DM have given conflicting results. Plasma Hcy levels have been reported to be increased, unchanged or decreased in T2DM.<sup>7-9</sup> In the present study, we confirmed that plasma Hcy concentration in T2DM was significantly higher than in healthy subjects. Conflicting results may relate to heterogeneity of the patients included, particularly with regard to folate, renal function status and presence of vascular arterial disease, which are the most important determinants of plasma Hcy level. Another important reason for conflicting results may relate to the remarkably small numbers of patients included in the studies assessing circulating Hcy levels in patients with diabetes. Other aspect that may explain the controversy may relate to the opposing influences on circulating Hcy levels exercised by the diabetic status per se, and by a number of risk factors that relate to or are accentuated by diabetes.<sup>6</sup> A recent human study has demonstrated that pathways of Hcy disposal are impaired in T2DM.<sup>26</sup> Studies have shown that the diabetic status may reduce rather than increase the circulating Hcy levels due to enhanced Hcy catabolism through the transsulfuration pathway,<sup>27</sup> because activities of cystathionine  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase, enzymes involved in the transsulfuration pathway were increased by the diabetic status.<sup>27-28</sup> While, cell culture have demonstrated that insulin mediates a decrease in the activities of MTHFR and CBS in HepG2 cells.<sup>28-29</sup> Ndrepepa *et al.* showed that circulating levels of folate and vitamin B<sub>12</sub> continue to be independent predictors of Hcy levels in T2DM.<sup>6</sup> Therefore, elevated Hcy levels in T2DM cannot be explained by the influence of diabetes itself, more attention should be focused on the influence of the diabetes-associated risk factors on Hcy metabolism.

N-3 PUFA is essential for normal growth and development and play an important role in the prevention and treatment of coronary heart disease,<sup>30-31</sup> hypertension,<sup>32</sup> and type 2 diabetes.<sup>13</sup> Our previous studies reported that plasma PL 20:5n-3, 22:6n-3, n-3:n-6, n-3 PUFA in the diabetes patient group were significantly lower, while plasma PL MUFA and n-6 PUFA were significantly higher when compared with a healthy control group.<sup>12</sup> In addition, platelet/plasma PL n-3 PUFA was negatively associated with plasma Hcy in middle aged and geriatric hyperlipaemia patients, and in healthy male subjects.<sup>14-15</sup> In the present study, we also found that plasma PL n-3:n-6 PUFA was negatively associated with plasma Hcy in T2DM. Plasma PL 22:6n-3 was negatively associated with plasma Hcy in healthy subjects.

Several randomized placebo-controlled clinical trials of small sample size and short duration have documented the effects of n-3 PUFA on plasma Hcy in diabetic dyslipidemia,<sup>33</sup> in acute myocardial infarction patients,<sup>34</sup> and in hyperlipidaemic men.<sup>35</sup> However, only one randomized double-blind, placebo-controlled clinical trial was conducted on T2DM. This study reported that the consumption of n-3 PUFA supplements (3 g/day) for 2 months decreases the levels of Hcy in diabetic patients.<sup>16</sup> Our previous meta analysis, which was based on the in-

**Table 3.** Partial correlations between plasma phospholipids fatty acid compositions and homocysteine and vitamin B group in type 2 diabetes and in healthy subjects, controlled for confounding factors

Fatty acids	Type 2 diabetes						Healthy subjects					
	Homocysteine		Folate		Vitamin B-12		Homocysteine		Folate		Vitamin B-12	
	Std Coef	<i>p</i> -value	Std Coef	<i>p</i> -value	Std Coef	<i>p</i> -value	Std Coef	<i>p</i> -value	Std Coef	<i>p</i> -value	Std Coef	<i>p</i> -value
16:00	0.269	0.026	0.052	0.676	-0.105	0.395	0.005	0.968	-0.099	0.386	-0.029	0.800
18:1n-9	0.246	0.043	-0.053	0.668	-0.112	0.362	0.210	0.041	-0.074	0.511	-0.172	0.125
18:1n-7	0.288	0.017	0.102	0.406	-0.188	0.124	0.033	0.773	-0.014	0.904	-0.014	0.903
18:3n-3	0.137	0.266	-0.007	0.956	-0.089	0.469	-0.073	0.520	0.150	0.186	-0.068	0.551
20:4n-6	0.303	0.012	-0.003	0.981	0.241	0.048	0.002	0.985	-0.100	0.383	-0.103	0.365
20:5n-3	0.018	0.884	-0.024	0.847	-0.142	0.470	0.023	0.839	-0.015	0.893	0.055	0.633
22:4n-6	0.252	0.038	0.060	0.629	-0.171	0.164	0.057	0.621	-0.030	0.792	0.015	0.898
22:5n-6	-0.466	<0.001	-0.140	0.253	0.009	0.941	-0.082	0.474	0.159	0.161	0.234	0.038
22:5n-3	0.312	0.010	-0.051	0.681	-0.168	0.170	-0.048	0.676	0.022	0.847	-0.084	0.463
22:6n-3	-0.046	0.708	0.131	0.288	-0.053	0.667	-0.201	0.042	-0.078	0.488	0.193	0.044
Total SFA	0.147	0.231	0.017	0.893	-0.067	0.589	0.047	0.679	-0.154	0.170	-0.033	0.768
Total MUFA	0.238	0.050	-0.018	0.883	-0.082	0.504	0.074	0.512	0.025	0.823	-0.065	0.567
Total PUFA	0.303	0.012	0.002	0.987	0.117	0.343	-0.133	0.238	0.099	0.377	0.110	0.326
n-3 PUFA	-0.028	0.280	0.021	0.867	-0.116	0.345	-0.097	0.390	-0.026	0.819	0.135	0.228
n-6 PUFA	0.261	0.032	-0.013	0.917	0.174	0.156	-0.069	0.543	0.122	0.277	0.018	0.874
n-3:n-6	-0.400	0.046	-0.013	0.915	-0.111	0.369	-0.029	0.799	-0.064	0.569	0.091	0.420

Coef, partial correlation coefficient

The associations of plasma PL fatty acids compositions with plasma Hcy concentration was tested by using Pearson partial correlation analysis after controlling confounding factors (Age, sex, BMI).

consistent clinical trial results, demonstrated that high consumption of n-3 PUFA decrease plasma Hcy.<sup>36</sup>

However, the possible mechanism on how n-3 PUFA regulates Hcy metabolism is not fully understood at present. Piolot reported the apparent interaction of n-3 PUFA and NO on Hcy metabolism in healthy people.<sup>37</sup> They also suggested a probable mechanism by which n-3 PUFA supplementation can reduce the production of Hcy. The reduced Hcy concentrations observed in their study are attributed to possible oxidative stress induction by n-3 PUFA and stimulation of the oxidative catabolism of Hcy.<sup>38</sup> This increased susceptibility to oxidative stress due to n-3 PUFA supplementation is reported.<sup>39</sup> Furthermore, our animal study suggested that 22:6n-3 decrease plasma Hcy concentration through regulation of critical gene expression and enzyme activity.<sup>17</sup> Our population studies found that dietary fatty acids interact with methylenetetrahydrofolate reductase (MTHFR) and methionine adenosyltransferase I, alpha (MAT1A) genetic variants in determining plasma Hcy concentration.<sup>18-19</sup> Considering the significance of n-3 PUFA in protecting CVD, more experiments based on animal study, cell culture and population study should be conducted to investigate the potential mechanism by which n-3 PUFA decrease plasma Hcy. In conclusion, increased plasma Hcy levels in T2DM associated with low n-3:n-6 ratio intake. We suggest that T2DM increase their long chain n-3 PUFA intake from fish or fish oil while decreasing n-6 PUFA intake.

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#### AUTHOR DISCLOSURES

All authors read and approved the final manuscript. The work undertaken is not owned by nor has it been conducted for any for-profit entity. The authors have no financial interest in this work.

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## Original Article

## Plasma phospholipids polyunsaturated fatty acids and plasma homocysteine in Chinese type 2 diabetes patients

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### 中国二型糖尿病患者血浆磷脂多元不饱和脂肪酸与血浆同型半胱氨酸

本研究的主要目的是探究中国二型糖尿病患者血浆磷脂多元不饱和脂肪酸与血浆同型半胱氨酸的关系。我们收集了 104 份二型糖尿病病例(年龄: 57.3±13.4 岁)和 150 个健康样本(年龄: 48.4±8.7 岁)。应用标准方法检测血浆的磷脂脂肪酸和同型半胱氨酸浓度。二型糖尿病患者血浆同型半胱氨酸浓度显著高于健康人的浓度( $p<0.001$ )。高同型半胱氨酸血症的发病率(36.54%)也显著高于健康人群(17.32%)( $p=0.012$ )。二型糖尿病患者的血浆磷脂所含的 20:4n-6( $r=0.303$ ,  $p=0.012$ )、22:5n-3( $r=0.312$ ,  $p=0.01$ )、总多元不饱和脂肪酸(PUFA)( $r=0.303$ ,  $p=0.012$ )、n-6 PUFA( $r=0.261$ ,  $p=0.032$ )与血浆同型半胱氨酸呈显著正相关;而血浆磷脂中的 n-3:n-6 PUFA( $r=-0.400$ ,  $p=0.046$ )与同型半胱氨酸呈显著负相关。健康人群血浆磷脂之 22:6n-3( $r=-0.201$ ,  $p=0.042$ )与血浆同型半胱氨酸亦呈显著负相关。另外, 血浆磷脂所含的 22:6n-3( $r=0.193$ ,  $p=0.044$ )和 22:5n-6( $r=0.234$ ,  $p=0.038$ )与血浆维生素 B-12 呈显著负相关。我们的研究表明, 二型糖尿病患者高同型半胱氨酸浓度与较低的血浆磷脂 n-3:n-6 相关。我们建议二型糖尿病患者应该通过食用鱼和鱼油来增加饮食中的 n-3 PUFA 的摄入, 而降低 n-6 PUFA 的摄入。

**关键词:** n-3 多元不饱和脂肪酸、同型半胱氨酸、二型糖尿病、磷脂、单元不饱和脂肪酸