Low circulating dihomo-gamma-linolenic acid is associated with diabetic retinopathy: a cross sectional study of KAMOGAWA-DM cohort study

Takuro Okamura¹⁾, Hanako Nakajima¹⁾, Yoshitaka Hashimoto¹⁾, Saori Majima¹⁾, Takafumi Senmaru¹⁾, Emi Ushigome¹⁾, Naoko Nakanishi¹⁾, Masahide Hamaguchi¹⁾, Mai Asano¹⁾, Masahiro Yamazaki¹⁾, Hiroshi Takakuwa²⁾ and Michiaki Fukui¹⁾

¹⁾ Department of Endocrinology and Metabolism, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

²⁾ Agilent Technologies, Chromatography Mass Spectrometry Sales Department, Life Science and Applied Markets Group, Tokyo 192-8510, Japan

Abstract. Diabetic retinopathy (DR), one of the major complications of diabetes, can cause blindness and reduce quality of life. Dyslipidemia is reported to be associated with DR, whereas arachidonic acid may have a protective effect against DR. We aimed to investigate the association of circulating n-3 and n-6 polyunsaturated fatty acids (PUFAs) with DR. In this cross-sectional study, 190 Japanese patients with type 2 diabetes were classified as no diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), or proliferative diabetic retinopathy (PDR) including pre-proliferative diabetic retinopathy. Circulating fatty acids (FAs) were measured by gas chromatograph-mass spectrometry. Logistic regression analysis was performed to investigate the association between the levels of FAs and the presence of DR. The average age, body mass index and the duration of diabetes were 62.7 ± 12.1 years, 25.0 ± 4.5 kg/m², and 9.8 ± 8.7 years, respectively. Twenty-seven patients were diagnosed with DR. Circulating levels of dihomo-gamma-linolenic acid (DGLA) in the NDR ($n = 163$), SDR ($n = 13$) and PDR ($n = 14$) groups were 28.3 ± 11.0 µg/mL, 24.4 ± 9.7 µg/mL, and 21.8 ± 6.2 µg/mL, respectively ($p = 0.032$). The logarithm of circulating DGLA levels was associated with the presence of DR after adjusting for covariates (OR of 1-unit increment: 0.79, 95% CI: 0.62–1.00, $p = 0.049$). Circulating DGLA was negatively associated with the presence of DR.

Key words: Cohort study, Epidemiology, Fatty acids, Dihomo-gamma-linolenic acid, Diabetic retinopathy

DIABETIC RETINOPATHY (DR), a common micro-vascular complication of diabetes that causes irreversible retinal damage, is the leading cause of vision loss among working-age adults. Approximately one-third of patients with diabetes have some degrees of DR, and one in ten will develop a vision-threatening disease [1, 2]. Therefore, the prevention and treatment of DR is one of the most important issues in the treatment of diabetes.

Dyslipidemia is correlated with the progression of DR in humans [3]. In the Early Treatment Diabetic Retinopathy Study, dyslipidemia increased the risk of developing hard exudates in the macula and visual loss [4]. Excess free fatty acid metabolism results in dyslipidemia, and fatty

acids (FAs) are also involved in inflammation and related chronic diseases [5, 6]. Circulating FAs are composed of saturated FAs with no double bonds, monounsaturated FAs with a single double bond, and polyunsaturated FAs (PUFAs) with multiple double bonds. The association between FAs and DR has been reported in several studies, with early research suggesting a protective role of PUFAs against DR [7, 8], and recent reports associating n-3 PUFAs with a reduced risk of DR [9, 10]. In several animal studies, an n-3 PUFA-enriched diet was protective of retinal neuron function [11, 12]. While a protective role of n-3 PUFAs against DR has been observed, there has been no significant association reported to date between n-6 PUFAs and DR. Gamma-linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA) have been shown to induce T-regulatory cell activity and reduce the production of proinflammatory interleukin-1 and tumor necrosis factor- α [13]. In addition, moderate intakes of dietary n-6 PUFAs have reduced the risk of coronary heart disease [14], while circulating n-6 PUFAs

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Correspondence to: Yoshitaka Hashimoto, Department of Endocrinology and Metabolism, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, 465, Kajii-cho, Kamigyo-ku, Kyoto City, Kyoto 621-8585, Japan.

E-mail: y-hash@koto.kpu-m.ac.jp

are reported to be inversely associated with total and coronary heart disease mortality [15]. Taken together, not only n-3 PUFAs but also n-6 PUFAs have demonstrated anti-inflammatory effects.

In recent years, it is clearly shown that of patients with diabetes have different circulating FAs compared to those in people without diabetes, which may contribute to the development of organ damages [16–18]. Nevertheless, the association between these circulating FAs, especially detailed composition of FAs, and diabetic retinopathy has remained unclear. Therefore, this cross-sectional study investigated the association between circulating n-3 or n-6 PUFAs and DR.

Materials and Methods

Study design and participants

The KAMOGAWA-DM study is an ongoing prospective cohort study that began in 2014 and includes the outpatient clinics of the Kyoto Prefectural University of Medicine (Kyoto, Japan) and the Kameoka Municipal Hospital (Kameoka, Japan) [19]. For the present cross-sectional study, we included patients with T2DM whose circulating FAs were measured, and excluded those who were taking eicosatetraenoic acid supplements. T2DM was diagnosed at all participating institutions based on the criteria by the American Diabetes Association [20]. This study was approved by the Ethics Committee of each participating hospital (ERB-C-534, RBMR-E-466-5).

Data collection

Information about patients' background demographics (*i.e.*, age, sex, disease duration, and smoking habits) was gathered from their medical records. Blood pressure was measured in the outpatient clinics. After an overnight fast, venous blood samples were collected to measure fasting plasma levels of glucose, C-peptide, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, creatinine, and uric acids. The level of hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography and presented herein using the National Glycohemoglobin Standardization Program unit.

Diagnosis of DR was made by ophthalmologists based on the Davis classification: no diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), or proliferative diabetic retinopathy (PDR) that also includes pre-proliferative diabetic retinopathy. Patients with DR were defined as those with SDR or PDR.

Estimation and assessment of the patients' habitual food and nutrient intake

A brief-type self-administered diet history questionnaire (BDHQ) was used for the assessment of habitual food and nutrient intakes [21]. Details of the BDHQ were provided elsewhere [22]. Briefly, the BDHQ is a dietary recall to assess the respondent's dietary intakes of 58 items over the past month. Based on the Standard Tables of Food Composition in Japan, we estimated the intakes of total energy (kcal/day), carbohydrate (g/day), protein (g/day), and total fat (g/day) using the calculation algorithm of BDHQ [22].

Measurement of circulating free fatty acids

The composition of FAs in frozen serum samples was measured by gas chromatograph-mass spectrometry (GC/MS) with the Agilent 7890B/5977B system (Agilent Technologies, Santa Clara, CA, USA). Fatty acids compositions can be measured by several ways. In this study, we chose the serum, because it was the simplest method and could be applied clinically. Twenty-five μL of serum was methylated with a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan). The final product was loaded onto a Varian capillary column (DB-FATWAX UI; Agilent Technologies). The capillary column used for fatty acid separation was CP-Sil 88 for FAME (100 m with an inner diameter of 0.25 mm and a membrane thickness of 0.20 μm , Agilent Technologies). The column temperature was maintained at 100°C for 4 min, increased gradually by 3°C/min to 240°C, and maintained at 240°C for 7 min. The sample was injected in the split mode with a split ratio of 5:1. Each fatty acid methyl ester was detected in selected ion monitoring mode. All results were normalized to the peak height of the internal standard C17:0 [23]. Average CV was 0.0245% (range 0.004–0.039%) ($n = 3$) for intra-day precision during a single analytical run and average CV 0.00237% (0.0010–0.0033%) on two separated days ($n = 6$) for inter-day precision. Each analytical run included a blank sample (without internal standard), and standard concentrations for calibration, and replicate sets of QC samples: 0.56 $\mu\text{g/mL}$, 1.77 $\mu\text{g/mL}$, 11.22 $\mu\text{g/mL}$, and 60.00 $\mu\text{g/mL}$ and found to be linear from the concentrations of 0.56 to 60.00 $\mu\text{g/mL}$. ($y = 0.159x + 0.1392$).

Statistical analysis

The JMP program (ver. 13.0, SAS Institute, Cary, NC) was used for all statistical analyses. Differences with p values <0.05 were considered significant. Categorical variables were expressed as frequencies, and continuous variables were presented as mean $\pm$ standard deviation (SD). The Chi-square test for categorical variables and the Wilcoxon signed-rank test for continuous variables

were used to assess the differences between groups. The difference in each circulating FA level among patients with NDR, SDR, and PDR was compared by one-way ANOVA and Tukey's honestly significant difference test. Circulating DGLA levels were log transformed to create a more normal distribution. The odds ratio (OR) and 95% confidence interval (CI) of the logarithm of circulating DGLA levels and other variables based on the presence of DR were assessed by univariate and multivariate logistic regression analyses adjusting for sex, disease duration [24], systolic blood pressure, HbA1c [25], triglycerides, total cholesterol, high-density lipoprotein cholesterol [26], omega-6 fatty acids [27, 28], smoking status [29], total energy intake [30], and statin usage [31].

Results

Among 426 patients (234 males and 192 females) with T2DM who completed the BDHQ, circulating FAs were measured in 197 patients (96 males and 101 females). Seven patients were excluded due to the use of eicosapentaenoic acid supplements (Fig. 1).

The clinical characteristics of the 190 patients included in this study are summarized in Table 1. The average age and the level of HbA1c was 62.7 ± 12.1 years and $7.5 \pm 1.6\%$, respectively. The frequency (percentage) of patients with NDR, SDR, and PDR was 163 (85.8%), 13 (6.8%), and 14 (7.4%), respectively.

The differences of circulating PUFAs among diabetic retinopathy were evaluated (Table 2, Fig. 2). There was a difference of DGLA levels among retinopathy.

DGLA was negatively associated with the presence of DR in both the univariate logistic regression analysis (OR of 1-unit increment: 0.76, 95% CI: 0.62–0.96, $p = 0.020$) and multivariate logistic regression analysis after

adjusting for covariates (OR of 1-unit increment: 0.73, 95% CI: 0.53–0.99, $p = 0.043$) (Table 3).

Table 1 Clinical characteristics of the study patients

	Total ($n = 190$)
Sex, men/women	93/97
Age, yrs	62.7 (12.1)
Duration of diabetes, yrs	9.8 (8.7)
BMI, kg/m ²	25.0 (4.5)
Systolic blood pressure, mmHg	135.6 (19.1)
Diastolic blood pressure, mmHg	81.8 (10.7)
Fasting plasma glucose, mmol/L	7.2 (1.6)
Hemoglobin A1c, %	7.5 (1.6)
C-peptide, nmol/L	0.6 (0.3)
Triglycerides, mmol/L	1.5 (0.9)
Total cholesterol, mmol/L	5.2 (1.0)
High-density lipoprotein cholesterol, mmol/L	1.5 (0.5)
Low-density lipoprotein cholesterol, mmol/L	3.0 (0.7)
Creatinine, μ mol/L	70.9 (22.0)
Estimated GFR, mL/min/1.73 m ²	70.4 (18.5)
Uric acids, mmol/L	309.8 (77.7)
Urine albumin to creatinine ratio, mg/gCr	202.0 (583.0)
Diabetic nephropathy	
Normoalbuminuria	115 (60.5)
Microalbuminuria	49 (25.8)
Macroalbuminuria	26 (13.7)
Diabetic retinopathy	
NDR	163 (85.8)
SDR	13 (6.8)
PDR	14 (7.4)
Diabetic neuropathy –/+	150/40
Never smoker	120 (63.2)
Ex-smoker	39 (20.5)
Current smoker	31 (16.3)
Energy intake/day, kcal	1,682.5 (587.3)
Carbohydrate intake, g/day	209.9 (80.6)
Protein intake, g/day	71.7 (28.6)
Total fat intake, g/day	53.5 (20.0)

Data are expressed as number (%) of subjects or mean (SD).

BMI, body mass index; GFR, glomerular filtration rate; NDR, no diabetic retinopathy; PDR, proliferative diabetic retinopathy; SDR, simple diabetic retinopathy.

Fig. 1 The registration of patients.

Table 2 Circulating PUFAs of the study patients

Fatty acids, µg/mL	All (<i>n</i> = 190)	NDR (<i>n</i> = 163)	SDR (<i>n</i> = 13)	PDR (<i>n</i> = 14)	<i>p</i> -value
Linoleic acid (C18:2n6)	868.1 (370.9)	875.9 (363.5)	792.5 (538.3)	847.6 (281.1)	0.723
γ-Linolenic acid (C18:3n6)	5.2 (4.7)	5.3 (4.8)	6.1 (3.5)	3.6 (4.3)	0.347
Dihomo-γ-linolenic acid (C20:3n6)	27.6 (10.7)	28.3 (11.0)	24.4 (9.7)*	21.8 (6.2)*,†	0.032
Logarithm of dihomogamma-linolenic acid	8.24 (2.23)	8.40 (2.27)	7.58 (2.10)*	7.05 (1.36)*,†	0.049
Arachidonic acid (C20:4n6)	157.6 (69.6)	160.4 (68.6)	165.6 (68.6)	117.1 (75.2)	0.074
α-Linolenic acid (C18:3n3)	22.2 (10.4)	22.4 (10.7)	20.7 (8.2)	21.9 (8.9)	0.842
Eicosatrienoic acid (C20:3n3)	0.1 (0.9)	0.1 (0.9)	0.0 (0.0)	0.0 (0.0)	0.921
Eicosapentaenoic acid (C20:5n3)	42.0 (24.5)	42.8 (29.0)	32.1 (14.7)	41.6 (14.4)	0.402
Docosahexaenoic acid (C22:6n3)	146.1 (63.9)	148.1 (65.4)	130.0 (68.6)	138.0 (36.9)	0.551

Data are expressed as mean (SD).

p values by one-way analysis of variance analyses. The analyses of circulating PUFA levels among three groups were performed by Tukey HSD test: *, *p* < 0.05 versus NDR; †, *p* < 0.05 versus SDR.

NDR, no diabetic retinopathy; PDR, proliferative diabetic retinopathy; PUFAs, polyunsaturated fatty acids; SDR, simple diabetic retinopathy.

Fig. 2 Circulating PUFAs of the study patients

The analyses of circulating PUFA levels among three groups were performed by one-way ANOVA; *, *p* < 0.05.

NDR, no diabetic retinopathy; PDR, proliferative diabetic retinopathy; PUFAs, polyunsaturated fatty acids; SDR, simple diabetic retinopathy.

Discussion

In the present study of 190 Japanese individuals with T2DM from the KAMOGAWA-DM cohort study, we investigated the association between circulating n-3 or n-6 PUFAs and DR, and revealed that circulating DGLA

is negatively associated with the presence of DR.

Possible explanations of the inverse association between circulating DGLA levels and the presence of DR were explored. DGLA, an essential fatty acid, is n-6 PUFA with 20 carbons derived *in vivo* from linolenic acid. DGLA is converted to another n-6 PUFA with 20

Table 3 Logistic regression analyses for diabetic retinopathy

	Univariate		Multivariate	
	Odds ratio (95% CI)	<i>p</i> -value	Odds ratio (95% CI)	<i>p</i> -value
Men	1.15 (0.51–2.59)	0.745	1.54 (0.46–5.16)	0.484
Disease duration, yrs	1.05 (1.01–1.10)	0.015	1.04 (0.99–1.10)	0.117
Systolic blood pressure, mmHg	1.02 (0.99–1.04)	0.100	1.02 (0.99–1.04)	0.143
Hemoglobin A1c, %	1.35 (1.08–1.69)	0.009	1.23 (0.94–1.61)	0.145
Triglycerides, mmol/L	0.62 (0.34–1.14)	0.124	0.71 (0.29–1.75)	0.447
Total cholesterol, mmol/L	0.83 (0.55–1.25)	0.371	0.84 (0.49–1.46)	0.552
HDL-cholesterol, mmol/L	1.23 (0.53–2.88)	0.631	0.91 (0.25–3.30)	0.888
Omega-6 fatty acids, µg/mL	0.99 (0.99–1.00)	0.425	1.00 (0.99–1.00)	0.335
Never smoker	1.00 (Reference)	—	1.00 (Reference)	—
Ex-smoker	1.22 (0.37–4.04)	0.746	2.28 (0.64–8.19)	0.205
Current smoker	2.84 (1.13–7.13)	0.026	0.92 (0.20–4.12)	0.912
Total energy, kcal/day	1.00 (0.99–1.00)	0.571	0.84 (0.49–1.46)	0.889
Statin usage	2.08 (0.87–4.93)	0.098	2.15 (0.76–6.03)	0.147
Log dihomo-gamma-linolenic acid	0.76 (0.62–0.96)	0.020	0.72 (0.53–0.99)	0.038

Multivariate analysis was adjusted for was adjusted for sex, disease duration, systolic blood pressure, hemoglobin A1c, triglycerides, total cholesterol, high-density lipoprotein cholesterol, omega-6 fatty acids, smoking status, total energy, and statin usage.

carbons, arachidonic acid (AA) [32, 33]. Both DGLA and AA are substrates of the lipid-peroxidizing enzyme cyclooxygenase (COX) which metabolizes DGLA and AA to various bioactive metabolites, such as the 1 and 2 series prostaglandins (PGs1 and PGs2), through a series of free radical reactions. PGs2 are generally known as pro-inflammatory metabolites, whereas PGs1 possess anti-inflammatory effects [34]. Prostaglandins E1 (PGE1), which is a type of PGs1, could inhibit the proliferation of vascular smooth muscle cells and the adhesion of vascular cells, and the development of atherosclerosis [35, 36]. Furthermore, DGLA is metabolized by the 15-lipoxygenase into 15-(S)-hydroxy-8,11,13-eicosatrienoic acid (15-HETrE) [37], which has been reported to reduce the development of DR because it inhibits the activity of vasoconstrictors and metabolites of AA including thromboxanes (TXs) and leukotrienes (LTs) [38, 39]. Reduced plasma and vitreal concentrations of PGE1, as well as increased levels of TXs and LTs have been reported in patients with DR [40–42]. Therefore, the imbalance between the vasodilators and platelet anti-aggregators (*e.g.* PGE1) and the vasoconstrictors and platelet aggregators (*e.g.* TXs and LTs) may lead to ischemic vasoconstriction of retinal vessels and enhanced platelet aggregation.

Thus, the oxidative metabolites of DGLA, including PGE1 and 15-HETrE, have demonstrated clinical effi-

cacy in a variety of diseases including suppressing chronic inflammation [37], promoting vasodilation [43], lowering blood pressure [44], and inhibiting smooth muscle cell proliferation associated with atherosclerotic plaque development [45]. In this study, circulating levels of DGLA in patients with DR were significantly lower than those without (patients with DR: 7.31 ± 1.74 , patients without DR: 8.40 ± 2.27 , $p = 0.018$). Moreover, the logarithm of circulating DGLA levels and the logarithm of urine albumin excretion tended to be negatively correlated with each other ($\beta = -0.100$, $p = 0.171$). As mentioned above, the oxidative metabolites of DGLA, including PGE1 and 15-HETrE, have ameliorative effects on various diseases, and a similar mechanism was thought to have occurred in nephropathy.

Our study has a strength, and that is that the results of fatty acid measurements are extremely robust. At first, all results were normalized to the peak height of the internal standard C17:0, and the results were quite robust. Average CV of three samples of intra-day was 0.0245% (0.004–0.039%) and average CV of six samples of inter-day precision was 0.00237% (0.0010–0.0033%) and linearity was from 0.56 µg/mL to 60.00 µg/mL (Supplementary Table 1). There are three major limitations in this study that could be addressed in future research. First, this study was a cross-sectional design, and further research should be conducted to establish the

causal relationship between low circulating DGLA levels and DR. Second, the sample size in this study was relatively small and future large-scale studies are warranted. Third, fatty acids compete with each other in the metabolism, absorption, and incorporation to cells, therefore, it was better to use the proportion of specific fatty acid to total fatty acids to total fatty acids. However, many previous reports have performed various assessments using absolute values of circulating fatty acids [46-49], and significant associations with various diseases have been shown in those reports. Fourth, omega-6 fatty acids are metabolized by a single metabolic pathway, and it is unclear why only DGLA was significantly associated with the presence of DR. This is a topic for future research.

In conclusion, this study was the first to demonstrate that circulating levels of DGLA in patients with DR were significantly lower than those without. It is important to conduct future prospective study to investigate the relationship between circulating DGLA levels and incident DR, and explore the mechanisms that contribute to the difference between dietary and circulating DGLA.

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