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Research Article

Serum Glucose-Dependent Insulinotropic Polypeptide (GIP) and Glucagon-Like Peptide-1 (GLP-1) in association with the Risk of Gestational Diabetes: A Prospective Case-Control Study

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Background. Defects in incretin have been shown to be related to the pathogenesis of type 2 diabetes. Whether such a deficiency happens in gestational diabetes mellitus (GDM) remains to be confirmed. We assessed the association of fasting glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) with GDM. We also studied the longitudinal circulation of these peptides during pregnancy and afterwards. *Methods*. 53 women with GDM (30 managed with diet only (GDM-diet) and 23 treated with insulin (GDM-insulin)) and 43 pregnant women with normal glucose tolerance (NGDM) were studied, with GIP and GLP-1 levels measured at 24–28 weeks (E1), prior (E2) and after (E3) delivery, and postpuerperium (E4). *Results*. Basal GIP was shown to be low in GDM groups compared to NGDM in E1, and in E4 for GDM-diet. GLP-1 was low in GDM groups during pregnancy and afterwards. At E1, serum GIP and GLP-1 were inversely associated with GDM and participants with lower levels of GIP (<0.23 ng/mL) and GLP-1 (<0.38 ng/mL) had a 6 (95% CI 2.5-14.5)- and 7.6 (95% CI 3.0-19.1)-fold higher risk of developing GDM compared with the higher level, respectively. In the postpuerperium, when there is a drop in β-cell function, participants with previous GDM (pGDM) presented lower GLP-1 (in both GDM subgroups) and lower GIP in GDM-diet subgroup compared to controls. *Conclusion*. There is an independent, inverse association between fasting incretins and higher risk of GDM. Furthermore, lowered levels of these peptides may play an important role in the abnormality of glucose regulation following pregnancy.

1. Introduction

The gastrointestinal tract is the largest endocrine organ in the body, generating hormones that have significant signalling and sensing important roles in regulating body weight and energy expenditure [1]. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are known incretin peptides secreted from the intestine in response to nutrient ingestion that stimulate insulin secretion together with hyperglycaemia [2, 3]. The physiological importance of islet-derived GLP-1 and GIP in insulin secretion has been previously studied [4], where their contribution to the regulation of β-cell mass is debated in the pathophysiology of type 1 and type 2 diabetes (T1&T2DM) [5, 6]. Impaired incretin effect has been reported in patients with T2DM, where it has been concluded that this deficit is the outcome of the diabetic state and not a primary pathogenic trait leading to T2DM [7]. Nevertheless, a study by Amato et al. [8] has suggested that fasting incretins play an important role in the pathophysiology of T2DM.

Pregnancy is a condition associated with the physiological and reversible expansion of β-cell mass in both animals and humans [9]. A study on the role of incretin peptides in islet adaptation to gestation, using incretin receptor knockout mice has revealed the significant role of GLP-1 in pregnancy-induced elevation of β-cell mass, mediated largely by local GLP-1 production in α-cells. However, that study also found that islet or K-cell-derived GIP is not essential

for pregnancy-associated expansion of β-cell mass [9]. Gestational diabetes mellitus (GDM) is the most common medical complication during pregnancy and is defined as diabetes diagnosed during the gestational period that is not clearly overt diabetes [10, 11]. Higher fasting GLP-1 level in patients with GDM compared to pregnant women with NGDM have been observed by Cypryk et al. [12], where a lower but not significantly GLP-1 level has been reported by Lencioni et al. [13].

There is however paucity in information with regard to fasting gut peptide levels in pregnancy. We hypothesised that GDM pregnancies compared to NGDM will demonstrate impaired fasting levels of these peptides throughout pregnancy as the lack of these peptides can result in gestational hyperglycaemia. Further, beyond the pregnancy, low fasting gut peptide levels can provide early pathophysiologic insight into the transformation of GDM to T2DM later in life despite apparent normalisation of glucose tolerance in GDM after puerperium.

2. Material and Methods

This study was carried out in the Women and Children's Health Complex, University Malaya Medical Center (UMMC). The protocol of the present study was approved by the University of Malaya Medical Centre (UMMC) Ethics Committee (Ethics Committee Reference Number: 1052.8). A cohort of 434 patients was initially recruited at the time of GDM diagnosis (24-28 weeks of gestation). However, only 96 patients including 53 subjects diagnosed with GDM and 43 normal glucose tolerance pregnant control (NGDM) were entered in the longitudinal study as we were unable to obtain a fasting blood sample of all initially recruited participants at scheduled examination points. Furthermore, those that developed any pregnancy complications such as late-diagnosed GDM, preeclampsia, high blood pressure, eclampsia, and preterm labour were omitted from the longitudinal assessment [14]. The fasting maternal samples were collected at four points: (E1) 24-28 weeks of pregnancy at the time of OGTT, (E2) prior to parturition, (E3) early postpartum (24 hours after parturition), and (E4) 2-6 months of postpuerperium [14]. GDM was diagnosed as fasting plasma glucose (FPG) (≥5.1) and 75 g OGTT plasma glucose (≥7.8) [15].

Fasting glucose (FG) levels were measured using the glucose oxidase method (ADVIA® 2400 Clinical Chemistry System, Siemens, USA). A fasting level of serum total GIP, active GLP-1 (amide form), C-peptide, and insulin was determined using magnetic bead-based multiplex immunoassay, human diabetes panel (Bio-Plex Pro™, 171A7001M, USA) according to the manufacturer's protocol. The lower limit of quantitation (LLOQ) for GIP and GLP-1 was 11.2 and 31.3 pg/mL (0.0112 and 0.0313 ng/mL), and upper limit of quantitation (ULOQ) for GIP and GLP-1 was 22,895 and 16,000 pg/mL (22.895 and 16 ng/mL), respectively. The intra-assay coefficient of variation of GIP, GLP-1, insulin, and C-peptide was 2.47, 4.04, 3.04, and 5.9, respectively, where the interassay coefficient of variation was 3.08, 5.46, 2.55, and 2.33, respectively.

Body mass index (BMI) (weight (kg)/height (m^2)) was measured at 24-28 weeks of gestation, prior to parturition, and postpuerperium. Homeostasis model assessment of β-cell function (HOMA-β) was calculated as $(\text{FI} \times 20) \div (\text{FG}-3.5)$. Insulin resistance index (HOMA-IR) was calculated by the formula $\text{HOMA-IR} = [\text{FI} \times \text{FG}]/22.5$ [16]. HOMA model was derived from fasting blood glucose (FG) and fasting insulin (FI).

2.1. Statistical Analysis. The results were reported as mean ± standard error (SE), where a Kolmogorov–Smirnov test was used to assess the normality of data. Differences between groups were analysed using the Student t-test or Mann–Whitney U test. One-way ANOVA or the Kruskal–Wallis test was used for between-group comparisons in cases of more than two independent groups. Repeated measures ANOVA or Friedman's test was applied for within-group comparisons. In cases of sphericity assumption violation in repeated measures ANOVA, the Greenhouse–Geisser adjustment was used. Bonferroni post hoc analysis was used for pairwise comparisons within groups. Bivariate Spearman or Pearson was applied to assess correlations. The mean was considered as a cut-off point value, and logistic regression models were performed to compute crude/adjusted odds ratios (OR/aOR) and 95% confidence intervals (95% CI) comparing the risk of GDM among the two halves for serum GIP and GLP-1 concentrations. Skewed variables were log-transformed for skewed data. Statistical analysis was performed using IBM SPSS 20.0.

3. Results

Fifty-three subjects diagnosed with GDM, and forty-three normal glucose tolerance pregnant women (NGDM) were included from the cohort in this study. At the time of enrolment (E1), no significant differences were observed in the mean of maternal ($p = 0.24$) and gestational age ($p = 0.72$), prepregnancy ($p = 0.40$), pregnancy BMI ($p = 0.88$), systolic (SBP) and diastolic (DBP) blood pressure, and family history of diabetes between the studied groups. Normal pregnancy presented higher HDL and LDL levels ($p = 0.02$) compared to GDM (Table 1). As was expected, GDM pregnancy presented a higher fasting glucose level (5.0 vs. 4.24, $p = 0.003$), 2-hour OGTT (10.8 vs. 5.9, $p = 0.005$), insulin resistance index (HOMA-IR) (2.9 vs. 2.1, $p = 0.03$), and lower HOMA-β (8.46 vs. 13.53, $p = 0.001$) compared to normal glucose tolerant subjects. There was no difference between insulin ($p = 0.72$) and C-peptide ($p = 0.39$) in both groups.

The results of between- and within-group comparisons of GDM and control groups (NGDM) are presented in Table 2. In the longitudinal assessment, pregnancy diagnosed with GDM presented a higher level of FG compared to NGDM. There were significant changes in FG levels in both groups; however, its level increased immediately after delivery and remained unchanged in postpuerperium. Pregnant women of both groups had statistically similar fasting insulin and C-peptide levels during all points of examination. In both groups, serum insulin and C-peptide levels rose during pregnancy, reached a peak in the late pregnancy, and then

Table 1: Baseline characteristics of participants (mean ± SE).

Participants' age (year)	
GDM	33.2 (0.6)
NGDM	32.1 (0.8)
Gestational age (week)	
GDM	25.8 (0.2)
NGD	25.9 (0.2)
Prepregnancy BMI (kg/m^2)	
GDM	26.1 (0.8)
NGDM	25.2 (0.7)
Pregnancy BMI (kg/m^2)	
GDM	29.3 (0.7)
NGDM	29.1 (0.9)
HOMA-IR	
GDM	2.9 (0.3)
NGDM	2.1 (0.2)*
SBP (mmHg)	
GDM	110.70 (1.14)
NGDM	109.40 (1.46)
DBP (mmHg)	
GDM	66.23 (1.19)
NGDM	2.26 (0.07)
HDL (mmol/L)	
GDM	2.05 (0.06)
NGDM	2.26 (0.07)*
LDL (mmol/L)	
GDM	2.59 (0.14)
NGDM	3.02 (0.11)*
Cholesterol (mmol/L)	
GDM	5.81 (0.14)
NGDM	6.11 (0.14)
TG (mmol/L)	
GDM	2.68 (0.36)
NGDM	2.14 (0.13)

*p value < 0.05 difference between groups.

decreased immediately after delivery. GDM groups presented lower HOMA-β compared to controls in E1 and E4.

Over the gestational period and postpuerperium, subjects diagnosed with GDM presented lower levels of GIP and GLP-1 compared to the control group, with the exception of E2 for GIP. In both pregnancy groups, concurrent with pregnancy development, GIP levels rose to hit their peak in late pregnancy, where levels decreased significantly after delivery and this reduction continued till postpuerperium. In contrast, GLP-1 levels decreased over the gestational period, and after delivery, this reduction continued in GDM subjects and then increased in postpuerperium.

Subsequently, in the third trimester (E2), the GDM group was divided into the GDM-diet subgroup ($n = 30$) and GDM-insulin subgroup ($n = 23$) (Table 3). Insulin treatment was initiated in GDM, where self-monitoring blood glucose indicated poor glycaemic control despite oral metformin therapy or diet. Pregnant women of the GDM-insulin subgroup had significantly higher FG compared to GDM-diet and NGDM in all examination points. In both GDM subgroups, FG levels decreased gradually over the pregnancy period, increased slightly in immediate postpartum, and then gradually decreased in postpuerperium. The level of insulin and C-peptide of all subgroups increased during pregnancy and gradually decreased after delivery. GDM-insulin subjects in E1 and both GDM subgroups in E4 presented lower HOMA-β compared to the normal group.

In the first examination, both GDM subgroups presented lower GIP and GLP-1 compared to NGDM. During the pregnancy, GIP levels increased in all subgroups, decreased gradually after delivery, and remained higher compared to the first examination. In postpuerperium, GIP level of the pGDM-diet group remained significantly low compared to the controls. In contrast with a significant decrease in the GLP-1 level of controls, GLP-1 level of each GDM subgroup remained unchanged during the gestational period. In immediate postpartum, GLP-1 level of pGDM subgroups decreased and then increased to the higher level; however, its levels remained statistically low compared to controls.

Regarding baseline variables (E1), serum GIP and GLP-1 were inversely associated with GDM. Participants with lower levels of GIP (<0.23 ng/mL) and GLP-1 (<0.38 ng/mL) had a 6- and 7.6-fold higher risk of developing GDM compared with the higher level, respectively. However, this relationship was relatively unchanged in GIP and became stronger in GLP-1 after adjustment for confounders including maternal age, gestational age, and BMI (aOR 5.7, 95% CI 2.3-14.3). Lower levels of β-cell function index (HOMA-β) were also inversely associated with the risk of GDM (0.88 (95% CI 0.81-0.96)) (Table 4). GLP-1 was directly correlated with GIP ($r = 0.68$, $p < 0.001$).

4. Discussion

In this study, we evaluated alterations in the basal level of serum glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) levels in women with and without GDM, in an attempt to investigate the role of these peptides in glucose homeostasis during pregnancy and afterwards. In general, low basal circulating level of incretins is due to rapid degradation by dipeptidyl peptidase-4 (DPP-4) [17]; however, lower amounts of these peptides are essential for glucoregulation and their consequent inhibitory effect on pancreatic α-cells [17].

This study showed that basal GIP and GLP-1 concentration plays a significant role in glucose homeostasis. GLP-1 is primarily synthesized by L-cells in the gastrointestinal tract, where it is influenced by ingested glucose and fatty acids or stimulated vagus nerve stimulation [18]. GLP-1 stimulates insulin secretion by β-cells in the pancreatic islets and inhibits glucagon secretion by α-cells [19]. GIP is mainly secreted by K-cells (in the mucosa of the duodenum and jejunum and the proximal portion of the ileum) and stimulates food intake-mediated insulin secretion by pancreatic β-cells [20]. It has been shown that GIP increases insulin's effect by directly changing target tissue sensitivity to insulin [21].

Table 2: Between- and within-group comparisons of subjects with gestational diabetes mellitus (GDM, $n = 53$) vs. control group (NGDM, $n = 43$) (mean ± SE).

	E1	E2	E3	E4
FG (mmol/L)				
GDM	5.00 ± 0.22	4.66 ± 0.15	5.01 ± 0.18[b]	4.66 ± 0.08
NGDM	4.24 ± 0.06	4.36 ± 0.13	4.60 ± 0.12[a]	4.35 ± 0.08
p value	0.003	0.15	0.08	0.009
Insulin (ng/mL)				
GDM	0.45 ± 0.03	0.71 ± 0.09[a]	0.50 ± 0.08[b]	0.41 ± 0.03
NGDM	0.47 ± 0.06	0.61 ± 0.07[a]	0.40 ± 0.04[b]	0.45 ± 0.03
p value	0.72	0.40	0.34	0.28
HOMA-β				
GDM	8.46 ± 0.67	10.36 ± 1.45	6.23 ± 0.86	7.56 ± 0.62
NGDM	13.53 ± 1.45	14.92 ± 2.03	8.12 ± 1.28[a]	13.71 ± 1.75[c]
p value	0.001	0.17	0.18	0.001
C-peptide (ng/mL)				
GDM	1.13 ± 0.09	1.86 ± 0.16[a]	1.62 ± 0.21[b]	1.14 ± 0.07
NGDM	1.01 ± 0.10	1.44 ± 0.16[a]	1.25 ± 0.14	1.59 ± 0.28
p value	0.39	0.07	0.16	0.09
GIP (ng/mL)				
GDM	0.19 ± 0.01	0.54 ± 0.06[a]	0.36 ± 0.04[a,b]	0.29 ± 0.03[a,b]
NGDM	0.28 ± 0.01	0.53 ± 0.09[a]	0.53 ± 0.11[a]	0.38 ± 0.03[a]
p value	<0.001	0.57	0.007	<0.001
GLP-1 (ng/mL)				
GDM	0.34 ± 0.01	0.33 ± 0.01[a]	0.30 ± 0.01[a,b]	0.38 ± 0.02[b,c]
NGDM	0.47 ± 0.02	0.37 ± 0.01[a]	0.48 ± 0.01[b]	0.50 ± 0.01[b]
p value	<0.001	0.003	<0.001	<0.001

*p value < 0.05 difference between groups. [a]$p < 0.05$ compared to examination 1. [b]$p < 0.05$ compared to examination 2. [c]$p < 0.05$ compared to examination 3.

Furthermore, it has been suggested that GIP plays a role not only as an incretin hormone but also as a regulator of inflammation and insulin resistance [22]. GIP and GLP-1 share mutual characteristics as incretins, but they also possess distinct biological features [23]. Incretin peptides inhibit β-cell apoptosis and stimulate proliferation, resulting in the development of β-cell mass [17]. It has been shown that the transcription factor 7-like 2 (TCF7L2) gene is strongly associated with T2DM and GDM. Decreased TCF7L2 protein levels in T2DM correlated with the downregulation of GIP and GLP-1 receptors (GIP-R and GLP-1R), and impaired β-cell function [24, 25], lead to fasting and postprandial hyperglycaemia [26–28]. In the present study, we have shown that impaired fasting glucose at the time of GDM screening (24-28 weeks) is characterised by a reduced pancreatic β-cell function evaluated by HOMA-β and decreased insulin sensitivity evaluated by HOMA-IR. Interestingly, we found that low fasting levels of GIP and GLP-1 were inversely associated with higher risks of GDM in pregnancy. Pregnant subjects with a low level of GIP and GLP-1 were about 6- and 7.6-fold, respectively, at higher risk of GDM. As shown previously, GDM results from reduced pancreatic β-cell function [29]. Similarly, the importance of fasting incretin peptides has also been studied in patients with T2DM, and it was proposed that the reduced fasting incretin levels in the presence of worsening fasting glucose is secondary to weakened fasting β-cell function associated with increased α-cell activity [8]. The authors of that study suggested that early assessment of basal incretin level would be useful in the diagnosis of T2DM. A deficit in the regulation of these peptides has also been proposed, involved in the glucose homeostasis in T2DM, where it has been suggested as a novel possibility for treating subjects with T2DM [30].

In the longitudinal assessment of the present study, with progression in pregnancy, GLP-1 concentrations remained statistically unchanged in GDM groups, whereas its level decreased significantly in controls. The level of circulating GLP-1 did not differ between the GDM-insulin subgroup and nondiabetic pregnant women, whereas GDM-diet presented a lower level of this peptide compared to controls. This result indicates the effectiveness of exogenous insulin administration in the regulation of GLP-1 in GDM. Normal pregnancy is associated with insulin resistance and with pregnancy progression, where insulin sensitivity may gradually decline to 50% of the normal expected value [31, 32]. It has been proven that enlarged β-cell mass is an adaptation

Table 3: Between- and within-group comparisons of GDM-diet ($n = 23$) and GDM-insulin ($n = 30$) subgroup vs. control group (NGDM) ($n = 43$).

	Group	E1	E2	E3	E4
FBG (mmol/L)	GDM-diet	4.52 ± 0.11	4.37 ± 0.13	4.73 ± 0.21	4.62 ± 0.11
	GDM-insulin	5.67 ± 0.47*,†	5.05 ± 0.30*,†	5.41 ± 0.30*	4.70 ± 0.10^{c}
	NGDM	4.24 ± 0.06	4.36 ± 0.13	4.60 ± 0.12	4.35 ± 0.08
Insulin (ng/mL)	GDM-diet	0.45 ± 0.05	0.67 ± 0.14	0.50 ± 0.11	0.40 ± 0.03
	GDM-insulin	0.44 ± 0.04	0.77 ± 0.11^{a}	0.52 ± 0.14	0.41 ± 0.05^{b}
	NGDM	0.47 ± 0.06	0.61 ± 0.07	0.40 ± 0.04	0.45 ± 0.03
HOMA-β	GDM-diet	9.79 ± 0.83	10.75 ± 1.12	6.91 ± 1.38	7.77 ± 0.93*
	GDM-insulin	6.65 ± 1.01*	9.91 ± 2.27	5.27 ± 0.71	7.29 ± 0.80*
	NGDM	13.53 ± 1.45	14.92 ± 2.03	8.12 ± 1.28	13.71 ± 1.75
C-peptide (ng/mL)	GDM-diet	1.17 ± 0.13	1.82 ± 0.20^{a}	1.61 ± 0.23	1.12 ± 0.10^{b}
	GDM-insulin	1.07 ± 0.10	1.92 ± 0.25	1.64 ± 0.40	1.17 ± 0.11^{b}
	NGDM	1.01 ± 0.10	1.44 ± 0.16	1.25 ± 0.14	1.59 ± 0.28
GIP (ng/mL)	GDM-diet	0.20 ± 0.01*	0.54 ± 0.09^{a}	0.34 ± 0.05^{a}	0.24 ± 0.03^{b,*}
	GDM-insulin	0.18 ± 0.02*	0.54 ± 0.08^{a}	0.38 ± 0.08^{a}	0.34 ± 0.0 4^{a}
	NGDM	0.28 ± 0.01	0.53 ± 0.09	0.53 ± 0.11	0.38 ± 0.03
GLP-1 (ng/mL)	GDM-diet	0.36 ± 0.001*	0.34 ± 0.01	0.30 ± 0.01^{a,*}	0.39 ± 0.03*
	GDM-insulin	0.33 ± 0.011*	0.31 ± 0.01	0.29 ± 0.01^{a,b,*}	0.36 ± 0.03^{c,*}
	NGDM	0.47 ± 0.02	0.37 ± 0.01	0.48 ± 0.01	0.50 ± 0.01

Pairwise analysis comparison (Bonferroni post hoc adjustment) following one-way ANOVA or Kruskal–Wallis test. ^{a}p value < 0.05 compared to examination 1. ^{b}p value < 0.05 compared to examination 2. ^{c}p value < 0.05 compared to examination 3. $^{\dagger}p$ value < 0.05 compared to GDM-diet. $^{*}p$ value < 0.05 compared to control.

Table 4: Binary logistic regression analysis for the prediction of gestational diabetes mellitus ($n = 96$).

		GDM			
		No ($n = 43$) N (%)	Yes ($n = 53$) N (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
GLP-1 (ng/mL)					
Half 1	<0.38	10 (21.3)	37 (78.7)	7.6 (3.0-19.1)	11.5 (3.8-34.8)
Half 2	≥0.38	33 (67.3)	16 (32.7)	Referent	Referent
GIP (ng/mL)					
Half 1	<0.23	12 (24.5)	37 (75.5)	6.0 (2.5-14.5)	5.7 (2.3-14.3)
Half 2	≥0.23	31 (66.0)	16 (34.0)	Referent	Referent
p value				<0.001	0.001
HOMA-β					
Half 1	<9.04			2.64 (1.1-6.2)	2.48 (1.0-6.00)
Half 2	≥9.04			Referent	Referent
p value				0.027	0.044

OR (95% CI) adjusted for maternal age, gestational age, and BMI. $^{*}p$ value < 0.05.

to progressive insulin resistance that develops during gestational period [33]. In line with our findings, lower fasting GLP-1 level have been reported in association with GDM and T2DM [34, 35]. In contrast, no significant difference in basal GLP-1 level of GDM patients and controls has been reported previously [13, 36]; this discrepancy between results may be due to the small sample size that was used in these studies. Furthermore, GLP-1 concentrations of GDM subgroups showed a reduction immediately after delivery and then increased in the late postpuerperium. However, after delivery,

its level increased gradually and reached to the highest level in the late postpuerperium compared to women with GDM in their pregnancy. From this result, we postulate that low concentrations of GLP-1, disregarding the mode of treatment with diet or insulin during pregnancy, may indicate early abnormality of glucose regulation and progression to T2DM.

Furthermore, with progression in pregnancy, we observed a gradual progress in β-cell function (HOMA-β) occurring with insulin resistance progress in all subgroups. Furthermore, GIP levels increased with pregnancy advancement and reached a peak in late pregnancy. Our study postulates that elevations in basal GIP levels during pregnancy in GDM subgroups may play an effective role in controlling fasting glycaemia and insulin resistance. In the late postpuerperium, GIP continued to stay in the low level in women with previous GDM treated with diet but not in insulin-treated subgroup. Furthermore, we found that insulin treatment during GDM could control GIP levels in the postpuerperium period and reduce its contribution in the pathogenesis of T2DM. However, further research is warranted to investigate the effect of insulin treatment on fasting GIP level.

In summary, this study has presented longitudinal circulation levels of fasting GIP and GLP-1 during pregnancy, after parturition and postpuerperium. From the current study, we postulate that lower levels of these peptides play a major role in the increased risk of GDM and dysregulation of glucose after pregnancy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Clinical Study

The Association between Polymorphisms of Vitamin D Metabolic-Related Genes and Vitamin D_3 Supplementation in Type 2 Diabetic Patients

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Objective. To investigate the effect of single nucleotide polymorphisms (SNPs) of the key genes in vitamin D metabolic pathway on the serum 25(OH)D level after long-term vitamin D_3 supplementation and provide a theoretical basis for rational vitamin D_3 supplementation in diabetic patients with different genetic backgrounds. *Methods*. Patients with type 2 diabetes (T2DM) who met the inclusive criteria were given 800 IU of vitamin D_3 daily for 30 consecutive months. Serum 25(OH)D levels was measured at enrollment and every 6 months after enrollment. The average value of four-time measurements represented individual serum 25(OH)D level during vitamin D_3 supplementation. Multiplex TaqMan genotyping was used to determine the distribution of eight candidate SNPs in genes of DHCR7, CYP2R1, CYP27B1, CYP24A1, and VDR, which are key genes in the vitamin D metabolic pathway, in diabetic patients. *Results*. At baseline, the average serum 25(OH)D level was 22.71 ± 6.87 ng/mL, and 17.9% of patients had a ≥30 ng/mL level. During supplementation, the level of 25(OH)D increased significantly at each time point, and the average 25(OH)D level increased to 30.61 ± 5.04 ng/mL; however, there were 44.6% of patients whose serum 25(OH)D levels were still below 30 ng/mL. In the patients with CYP27B1 (rs10877012) G/T genotype, 71.79% achieved sufficient level of 25(OH)D, which was significantly higher than the other two genotypes ($P < 0.05$). Compared with those with T/T genotype, the RR of the patients with rs10877012 for <30 ng/mL level was 0.544 (95% CI: 0.291-0.917), and the RR after adjusting age and outdoor activity was 0.560 (95% CI: 0.292-0.970). *Conclusion*. The serum 25(OH)D level in patients with diabetes mellitus after long-term vitamin D_3 supplementation is associated with CYP27B1 polymorphism. Patients with rs10877012 G/T allele have a better response to vitamin D_3 supplementation. *Trial Registration*. This trial is registered with ChiCTR-IPC-17012657.

1. Introduction

Vitamin D has been found to be involved in a variety of public health-significant diseases including bone diseases [1], diabetes [2], cardiovascular diseases [3], cancer [4], and metabolic syndrome [4]. Due to its various extraosseous effects and the association between its deficiency and insulin resistance along with diabetes initiation, the serum vitamin D levels gain an extensive attention in the field of endocrinology [5]. A large number of cross-sectional studies have shown a negative correlation between vitamin D status and T2DM prevalence [2, 6]. While several longitudinal studies in Europeans [7], African-Americans [8], South Asians [9], and in China [10] still demonstrated low levels of serum

25(OH)D which could predict the risk of type 2 diabetes, vitamin D supplementation also reduced the incidence of diabetes accordingly [7, 10]. Consistent with this, there was evidence to show that vitamin D deficiency is significantly higher in diabetic patients than in normal population in China [11]. Recent studies find that vitamin D_3 supplementation improved insulin secretion in diabetic patients [12] and show beneficial effects in diabetic patients with poor glycemic control [13]. Maintaining adequate vitamin D level in the population will be an important strategy to reduce the incidence of diabetes. Many physicians have accepted that vitamin D can be a component of prescription for diabetic patients. However, before that, there is still an important question, in view of the potential hazard of excessive vitamin D_3 supplementation, what the level of vitamin D is sufficient for diabetic patients.

25(OH)D, the main circulating metabolite of vitamin D, is a biomarker to indicate the level of vitamin D in the body. The factors affecting circulating 25(OH)D level include sunshine exposure and dietary intake; however, its high heritability suggests that genetic factors also played important roles [14]. It is increasingly recognized that genetic factors influence serum 25(OH)D status. Previous studies based on European and Chinese twins and families have confirmed that genetic factors have a significant impact on the individual variation of 25(OH)D levels. The heritability is estimated to be as high as 53% [14, 15]. Although several rare Mendelian diseases can cause functional vitamin D deficiency [16–18], there are few studies to investigate the effects of common genetic variants on vitamin D status, especially in diabetic patients. If vitamin D status is associated with certain genotypes or SNPs, then some people may need higher or lower level of serum 25(OH)D than general level to minimize health risk. Whether individuals with genotypes known to influence the efficiency of vitamin D_3 supplementation may require particular ("personalized") recommendations with respect to optimizing vitamin D_3 supplementation in order to minimize adverse health outcomes.

According to previous candidate gene studies [19] and genome-wide association studies [20, 21], some of the common SNPs in vitamin D metabolic pathway genes are found to be associated with the level of circulating 25(OH)D in common status. These SNPs encode key metabolic enzymes include 25-hydroxylase (CYP2R1), 1-hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), 7-dehydrocholesterol reductase (DHCR7), and vitamin D receptor (VDR). CYP2R1 converts vitamin D to 25(OH)D. CYP27B1 activates 25(OH)D to 1-alpha,25-dihydroxy-cholecalcifero ($1,25(OH)_2D_3$). CYP24A1 inactivates 25(OH)D and $1,25(OH)_2D_3$. DHCR7 shunts vitamin D precursors toward cholesterol biosynthesis. VDR binds $1,25(OH)_2D_3$ to activate gene transcription and regulates vitamin D metabolism. A potentially important clinical question is whether the polymorphism of these genes affects the vitamin D_3 supplementation in diabetic patients with adequate vitamin D administration. In this study, we investigated the question in diabetic patients with long-term administration of vitamin D_3 supplements.

2. Materials and Methods

2.1. Study Design and Participants. This study was a randomized controlled trial conducted at primary healthcare outpatient clinics in Lishui city (latitude: 28° N), which was located in Zhejiang Province, southeastern China. Individuals aged 50 years and older with a diagnosis of T2DM according to the diabetes diagnostic criteria set by the WHO in 1998 [22] were screened from residents in the local community in Lishui. The main exclusion criteria were as follows: impaired renal function (estimated glomerular filtration rate < 30 mL/min/1.73m^2, calculated from serum creatinine using the modification of renal disease formula); hypercalcemia (serum calcium > 2.65 mmol/L) of any reason; urolithiasis; serum 25(OH)D > 60 ng/mL or calcitriol use; and major diseases including active cancer in the past five years, current acute inflammation, and other serious complications of diabetes. Withdrawal criteria for premature termination of the trial were as follows: serum 25(OH)D > 100 ng/mL, onset of hypercalcemia, hypersensitivity to cholecalciferol, and onset of urolithiasis.

Subjects received 800 IU vitamin D_3 capsules (Xingsha Pharmaceuticals (Xiamen) Co., Ltd., China) that were taken daily at any time during the day and continued for a period of 30 months (from February 2015 to August 2017). A total of 115 diabetic patients were included in the study (Figure 1, flow diagram), and all participants provided written informed consent. This study conformed to the principles set by the Declaration of Helsinki and was approved by the Ethics Committee of Soochow University (ESCU-20160001).

2.2. Anthropometric Measurements. Before vitamin D_3 supplementation, a standard questionnaire was used to collect information regarding participants' demographics (i.e., age, sex, and district), physical activity, diabetes treatment (oral antihyperglycemic agent, insulin, or neither), and past calcium supplementation.

Height and body weight were measured in light clothing, and body mass index (BMI) was defined as body weight divided by height squared (kg/m^2). Waist circumference was measured midway between the iliac crest and the lowest rib and hip circumference over the great trochanters. Then, waist-to-hip ratio (WHR) was calculated. Blood pressure was measured by a digital sphygmomanometer (HEM-7211, Omron, Japan) three times while participants were in the relaxed sitting position after 15 min of rest. There is a 5 min rest period between each measurement, and the mean value of the three measurements were used for analysis. After a 5 min or longer rest, pulse rate was measured using pulse palpation over a 30 s period.

2.3. Biochemical Measurements. Fasting blood samples were collected for quantification of metabolic parameters. Participants were instructed to take all regular medications except for diabetes medication and take no aspirin or nonsteroidal anti-inflammatory drugs for 48 hours before the visit except those medications that were taken regularly. Peripheral venous blood sample was collected from each participant in a 10 h overnight fasting state for biochemical analysis. Serum 25(OH)D concentration was determined

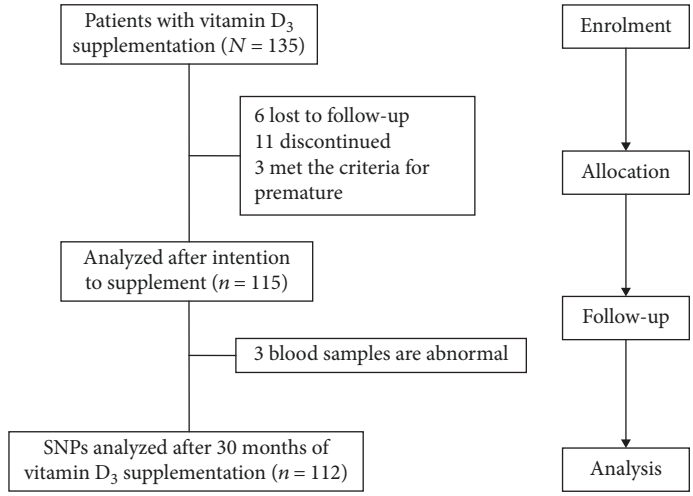

Figure 1: Flow diagram of study selection process.

by chemiluminescence immunoassay using an automatic chemiluminescence immunoassay analyzer (ADVIA Centaur XP, Siemens Healthcare diagnostics Inc., Tarrytown NY, USA). The intra and interassay coefficients of variation were 5.2% and 7.2% at the mean level of 28.2 ng/mL, respectively. According to the Endocrine Society clinical practice guideline [23], vitamin D_3 insufficiency and sufficiency were defined as serum $25(\mathrm{OH})\mathrm{D} < 30$ and ≥ 30 ng/mL. The concentrations of fasting blood glucose (FBG) and lipid profiles including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined with the use of enzymatic method on an automatic biochemistry analyzer (COBAS c702; Roche Diagnostics GmbH, Mannheim, Germany). Fasting insulin (FINS) levels were measured with electrochemiluminescence immunoassay on COBAS e601 (Roche Diagnostics GmbH, Mannheim, Germany). The homeostatic model assessment of insulin resistance (HOMA-IR) was computed by using the formula: $[\mathrm{FBG}\,(\mathrm{mmol/L}) \times \mathrm{fasting\ insulin}\,(\mathrm{mIU/L})]/22.5$. The levels of glycosylated hemoglobin (HbA1c) and high-sensitive C-reactive protein (Hs-CRP) were measured by immunoturbidimetry method (Roche Diagnostics GmbH, Mannheim, Germany). Abnormal standards for indicators such as blood glucose and blood lipids reference prevention guide standards of care for type 2 diabetes in China [24] and guidelines for the prevention and treatment of dyslipidemia in Chinese adults [25]. All blood samples were analyzed under blinded conditions. The lab tests were repeated at baseline and after 6, 12, 18, and 30 months.

2.4. SNP Selection and Genotyping. Eight candidate SNPs from five vitamin D pathway genes (CYP2R1, CYP27B1, CYP24A1, DHCR7, and VDR) associated with 25(OH)D or other health outcomes were selected by reviewing the literature on vitamin D_3 supplementation and metabolic gene correlation and the NCBI gene pool. The eight SNPs were CPY2R1 variant-rs10766197, CYP21R1 variant-rs 10741657, CYP27B1 variant-rs rs10877012, CYP27B1 variant-rs 4646536, CYP24A1 variant-rs 6013897, DHCR7 variant-rs 12785878, VDR variant-rs 2228570, and VDR variant-rs 1544410, respectively (Table S1).

Genomic DNA was extracted from 250 μL whole blood samples using Blood Genomic DNA Extraction Kit (Shanghai Jizhen Biological Technology Co., Ltd., Shanghai, China). The purity and concentration of Genomic DNA was detected by NanoDrop One Microvolume UV-vis spectrophotometer (Thermo Fisher Scientific, USA). And total DNA samples were diluted to the same concentration for downstream testing. Genotype analysis of key polymorphisms of vitamin D metabolic pathway was carried out using TaqMan® probes (Applied Biosystems Foster City, CA); PCR reactions were performed in 10 μL total volume, using 20 ng of DNA in TaqMan® Universal Master Mix with specific primers and probes (Applied Biosystems, Foster City, CA). The Bio-Rad CFX96 instrument equipped with the Bio-Rad CFX manager was used to assess the allelic content.

2.5. Statistical Analysis. Categorical variables were expressed as frequency (%), and continuous variables were expressed as mean ± standard deviation (SD). The serum 25(OH)D level of individuals after supplementation was represented by the average value of four-times tests during supplementation period. Chi-square or Fisher's exact test was used for the intergroup comparison of categorical variables. Paired t-test was used for the comparison of 25(OH)D level before and after supplementation. Chi-square was used to test whether the distribution of investigated SNPs meets Hardy-Weinberg equilibrium. Linkage disequilibrium test was measured using D'. The RRs for low level of 25(OH)D associated with various SNPs was estimated using log-binomial regression adjusted by age and outdoor activity. SNPStats software was used for Hardy-Weinberg equilibrium and linkage disequilibrium tests and SAS 9.3 software was used for other statistical analyses. All tests were two-side tests, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. General Characteristics of the Subjects. A total of 112 patients with diabetes were included in the SNPs analysis,

Table 1: Basic characteristics of diabetic patients.

Variable	Value (n (%) or mean ± SD)
Demographic indicators	
Sex (n, %)	
Male	31 (27.7)
Female	81 (72.3)
Age (y), mean ± SD	66.3 ± 9.1
Physical activity (h/day), n (%)	
<1 h	50 (44.6)
≥1 h	61 (55.4)
Calcium supplementation	
Yes	24 (21.4)
No	88 (78.6)
Region (n, %)	
Urban	48 (42.9)
Rural	64 (57.1)
Anthropometry	
Height (cm)	156.9 ± 6.7
Weight (kg)	61.5 ± 11.0
BMI (kg/m^2)	24.9 ± 3.9
Waist (cm)	85.8 ± 10.6
Hipline (cm)	95.7 ± 9.3
Waist-hipline ratio (cm/cm)	0.9 ± 0.1
Pulse (time/min)	75.8 ± 6.9
Systolic blood pressure (mmHg)	138.5 ± 15.9
Diastolic blood pressure (mmHg)	80.1 ± 8.6
Supplementary phase	25(OH)D (ng/mL)
Before supplementation	22.7 ± 6.9
Supplement for 6 months	29.2 ± 6.8*
Supplement for 12 months	29.0 ± 5.4*
Supplement for 18 months	28.9 ± 4.6*
Supplement for 30 months	35.3 ± 7.2*
Mean after supplementation	30.6 ± 5.0*

*Compared with before supplementation, $p < 0.05$.

including 31 males and 81 females, respectively. Compared with before supplementation, the 25(OH)D level of each detection time point was significantly increased after supplementation ($P < 0.05$), and the mean value of the four tests after supplementation was 30.61 ± 5.04 ng/mL. At baseline, 17.86% of patients had 25(OH)D ≥ 30 ng/mL, while the percentage increased to 55.40% after supplementation, and 44.60% of patients still had a 25(OH)D level below 30 ng/mL (Table 1). Except CYP27B1 (rs10877012 and rs4646536), other investigated genotypes such as CPY2R1 (rs10766197 and rs10741657), CYP24A1 (rs6013897), DHCR7 (rs12785878), and VDR (rs2228570 and rs1544410) all met the Hardy-Weinberg equilibrium. Two SNPs, rs10877012 and rs4646536, showed complete linkage disequilibrium (Table S1).

3.2. 25(OH)D Level and Insufficiency Risk in Patients with Different SNP Genotypes before and after Supplementation. Before supplementation, patients with different CYP24A1 (rs6013897) genotypes had different levels of serum 25(OH)D. All patients with A/A genotype had sufficient 25(OH)D. For other genes, different genotypes did not affect serum 25(OH)D level ($P > 0.05$) (Table S2). After supplementation, the 25(OH)D levels in the patients with different CYP27B1 genotypes (rs10877012 and rs4646536) are significantly different ($P < 0.05$). The proportion of patients with rs10877012 G/T genotype to achieve ≥30 ng/mL was 71.79%, while the proportions in other two genotypes were only 40%-50%. Relative to T/T, the RR in patients with G/T of rs10877012 to experience 25(OH)D insufficiency (<30 ng/mL) was 0.544 (95% CI: 0.291-0.917); after adjusting age, sex, and outdoor activity, the RR was 0.560 (95% CI: 0.292-0.970). For other genes, the 25(OH)D levels in the patients with different genotypes were not significantly different ($P > 0.05$) (Table 2).

3.3. Changes of 25(OH)D Levels in Patients with Different Genotypes of CYP27B1 (rs10877012) before and after Supplementation. There were significant differences in the 25(OH)D levels of TT, GG, and GT genotype in patients of baseline 25(OH)D < 30 ng/mL ($P = 0.045$), and patients with GT was significantly higher than patients with TT ($P = 0.017$). While there were no significant differences in the 25(OH)D levels of the three genotypes in patients with baseline 25(OH)D ≥ 30 ng/mL (Figure 2), in patients with baseline 25(OH)D < 30 ng/mL and ≥30 ng/mL, there was no significant difference in the increase of serum 25(OH)D levels after vitamin D_3 supplementation (Figure 3).

3.4. 25(OH)D Level and Insufficiency Risk in Patients with Different CYP27B1 (10877012) Genotypes after Stratification of Vitamin D Metabolism-Related Indicators. Relative to T/T, after adjusting age and outdoor activity, the RR of 25(OH)D insufficiency (<30 ng/mL) in female patients with CYP27B1 (rs10877012) G/T type was 0.423 (95% CI: 0.186-0.820); the RR of 25(OH)D insufficiency (<30 ng/mL) in overweight (BMI ≥ 24) patients with CYP27B1 (rs10877012) G/T type was 0.449 (95% CI: 0.312-0.809); the RR of 25(OH)D insufficiency (<30 ng/mL) in patients with abnormal total cholesterol (TC ≥ 5.20 mmol/L) with CYP27B1 (rs10877012) G/T type was 0.384 (95% CI: 0.087-0.494) (Table S3).

4. Discussion

Vitamin D deficiency and insufficiency has been involved in the development of diabetes. It gains much attention that whether vitamin D_3 supplementation is affected by genetic variation in key genes associated with vitamin D metabolism. In this study, we found that the SNPs of vitamin D metabolism-related genes rs10877012 and rs4646536 (completely linked) were correlated with serum 25(OH)D concentration after long-term adequate supplementation of 800 IU/day vitamin D_3 per day for 30 months in diabetic patients. In patients with insufficient 25(OH)D levels at

Table 2: 25(OH)D level and insufficiency risk in patients with different SNP genotypes after vitamin D_3 supplementation.

Gen	SNP	Gene locus	25(OH)D ≥ 30 ng/mL, *n* (%)	25(OH)D < 30 ng/mL, *n* (%)	RR (95% CI)[a]	P^b	RR (95% CI)[c]	P^d
CYP2R1	rs10766197	G/G	30 (60)	20 (40)	1	0.666	1	0.693
		A/A	8 (50)	8 (50)	1.25 (0.63, 2.18)		1.19 (0.60, 2.10)	
		A/G	24 (52.17)	22 (47.83)	1.19 (0.76, 1.91)		1.202 (0.76, 1.92)	
	rs10741657	G/G	22 (48.89)	23 (51.11)	1	0.244	1	0.294
		A/A	11 (73.33)	4 (26.67)	0.52 (0.17, 1.10)		0.54 (0.18, 1.15)	
		A/G	29 (55.77)	23 (44.23)	0.87 (0.56, 1.33)		0.87 (0.56, 1.32)	
CYP27B1	rs10877012	T/T	26 (48.15)	28 (51.85)	1	**0.031**	1	**0.046**
		G/G	8 (42.11)	11 (57.89)	1.12 (0.66, 1.71)		1.15 (0.67, 1.81)	
		G/T	28 (71.79)	11 (28.21)	**0.54 (0.29, 0.92)**		**0.56 (0.29, 0.98)**	
	rs4646536	C/C	26 (48.15)	28 (51.85)	1	**0.031**	1	**0.046**
		T/T	8 (42.11)	11 (57.89)	1.12 (0.66, 1.71)		1.15 (0.67, 1.81)	
		C/T	28 (71.79)	11 (28.21)	**0.54 (0.29, 0.92)**		**0.56 (0.29, 0.98)**	
CYP24A1	rs6013897	T/T	43 (57.33)	32 (42.67)	1	0.209	1	0.212
		A/A	2 (100)	0 (0)	—		—	
		A/T	17 (48.57)	18 (51.43)	—		—	
DHCR7	rs12785878	T/T	18 (60)	12 (40)	1	0.082	1	0.110
		G/G	23 (67.65)	11 (32.35)	0.81 (0.41, 1.58)		0.82 (0.41, 1.60)	
		G/T	21 (43.75)	27 (56.25)	1.41 (0.88, 2.47)		1.39 (0.87, 2.45)	
VDR	rs2228570	T/T	14 (53.85)	12 (46.15)	1	0.964	1	0.943
		C/T	11 (57.89)	8 (42.11)	0.97 (0.61, 1.68)		1.02 (0.64, 1.76)	
		C/C	37 (55.22)	30 (44.78)	0.91 (0.435, 1.77)		0.92 (0.44, 1.78)	
	rs1544410	G/G	5 (71.43)	2 (28.57)	1	0.367	1	0.404
		A/G	57 (54.29)	48 (45.71)	0.63 (0.12, 1.48)		0.66 (0.12, 1.52)	

Note: RR of 25(OH)D < 30 ng/mL. [a]Uncorrected RR. [c]Corrects age and amount of outdoor activity. *P* value is 25(OH)D < 30 ng/mL versus ≥30 ng/mL in comparison of all SNP genotypes. [b]Uncorrected RR. [d]Corrects age and amount of outdoor activity. Bold values are $p < 0.05$.

baseline, the 25(OH)D level of patients with rs10877012GT genotype was significantly higher than that of TT, but there was no significant difference in the proportion of increase after vitamin D_3 supplementation. Moreover, 71.79% patients carrying G/T genotype of rs10877012 achieved sufficient 25(OH)D levels (≥30 ng/mL), showing a better response to vitamin D_3 supplementation than those carrying T/T or G/G genotype. We found the phenomenon that heterozygote variants of rs10877012 are related to the effect of vitamin D supplementation through statistical analysis and not also one of the homozygotes. Similar to our results, Orton et al. reported that the associations of the heterozygous genotype CYP27B1 rs4646536 (C/T) with 25(OH)D level in a Canadian multiple sclerosis study [26]. However, the pattern of association between the alleles and 25OHD is not consistent in the other studies [21]. The inconsistency of this association may be due to the relatively small sample sizes in these studies or the different ethnicities.

The guideline of the Institute of Medicine (IOM) recommends an increase of vitamin D_3 intake of 200 IU/day compared with the guideline of 1997 [27]. Adults less than 70 years old need to meet a daily intake of 600 IU through recommended diets. For those above 70 years old, 800 IU is required per day [27]. IOM also increased the maximum safe dose of vitamin D_3 supplementation to 4000 IU per day [27].The supplementation dose used in our experiments was 800 IU/day which was considered to be sufficient. Our study suggested that after long-term supplementation, there was still about 50% patients whose 25(OH)D level did not reach the "ideal level" accepted by current standards (≥30 ng/mL). Genetic polymorphism affects patient's response to adequate vitamin D_3 supplementation; therefore, it is inappropriate to define a unique "ideal level" for peoples with different genetic backgrounds, e.g., for the diabetic patients carrying rs10877012 T/T and G/G genotype, ideal level of serum 25(OH)D may be lower than other SNPs. On the contrary, for diabetic patients with rs10877012 T/T or G/G genotype, especially women, overweight, and abnormally high-total cholesterol diabetes, vitamin D supplementation dose should be higher than that of the routine supplementary dose. These findings increase our understanding of vitamin D homeostasis and may help determine the benefit/risk of vitamin D_3 supplementation in Chinese diabetic patients.

Previous genome-wide association studies (GWAS) identified several SNPs in the vitamin D metabolic pathway associated with serum 25(OH)D status [20, 21]. However, few studies have investigated whether common genetic variants affect the response to vitamin D_3 supplementation. Several studies in a nondiabetic population have found rs10766197 (CYP2R1) [28], rs10741657 (CYP2R1) [29, 30], rs6013897 (CYP24A1) [29], rs2282679 (GC) [31], and rs7968585 (VDR) [29] were associated with the increase of serum

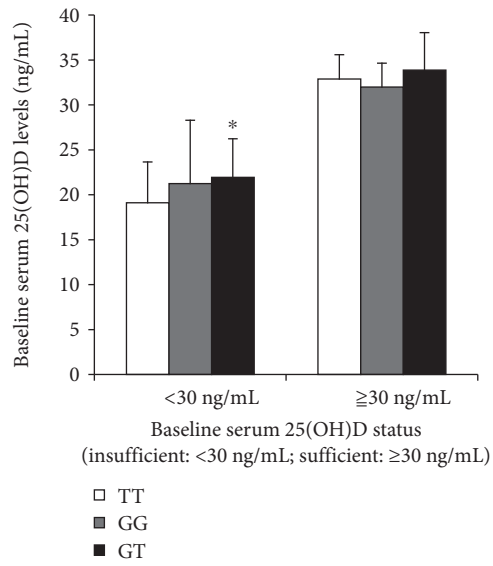

Figure 2: The levels of serum 25(OH)D in patients with different CYP27B1 (10877012) genotypes before vitamin D_3 supplementation. White bars: CYP27B1 rs10877012 TT genotype; gray bars: CYP27B1 rs10877012 GG genotype; black bars: CYP27B1 rs10877012 GT genotype. All data were expressed as means ± SD. $^*P < 0.05$.

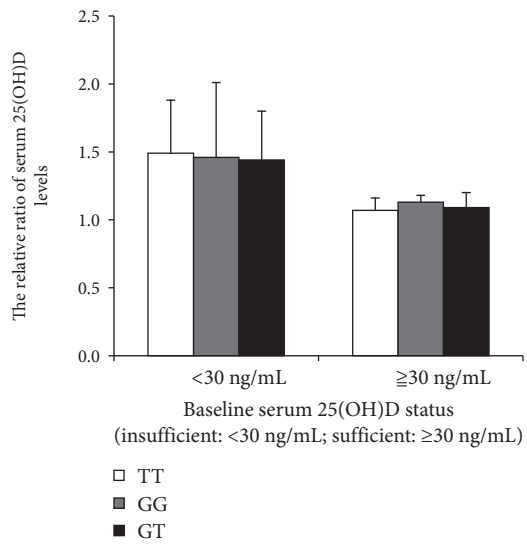

Figure 3: Comparison of serum 25(OH)D in the patients with different CYP27B1 rs10877012 genotypes before and after vitamin D_3 supplementation. The vertical axis is the relative ratio of serum 25(OH)D before and after 30 months of vitamin D3 supplementation. Insufficiency: serum 25(OH)D level of <30 ng/mL; sufficiency: serum 25(OH)D level of ≥30 ng/mL. There was no significant difference in the increase of serum 25(OH)D levels in patients of TT, GG, and GT genotype after supplementation. All P values were >0.05.

25(OH)D after vitamin D_3 supplementation. Similar studies were also conducted in gestational diabetes patients [32], but studies in patients with type 2 diabetes have not been reported. Our study found that although genetic variation of CYP27B1 was not significantly associated with baseline 25(OH)D level, it was associated with the response to vitamin D_3 supplementation. The published GWAS studies [20, 21] found that SNPs affecting baseline 25(OH)D were different from SNPs affecting the response to cholecalciferol supplementation, which was in line with our study. However, SNPs which have been reported to be associated with 25(OH)D in population cross-sectional and longitudinal studies, including DHCR7 (rs12785878) [33], CYP2R1 (rs10766197 and 10741657) [28, 34], CYP2 24A1 (rs6013897) [33, 35], and VDR (rs2228570 and 1544410) [26, 36], were not found to be associated with baseline 25(OH)D level or response to vitamin D_3 supplementation in our study. Further studies will elucidate effects of these SNPs on vitamin D metabolism.

CYP27B1 encodes 1a-hydroxylase that converts 25(OH)D to its active form, $1,25(OH)_2D_3$. CYP27B1 is a cytochrome P450 most strongly associated with the vitamin D status. The SNP rs10877012 that resides at position 1260 of CYP27B1 was widely explored for the association with 25(OH)D [19]. In a gestational diabetes study [37] and a large-scale cohort study [38], the C allele of rs10877012 was associated with lower level of 25(OH)D. The association of the rs10877012 C allele with low 25(OH)D level was also reported in an African-American study [39]. Although the effect of rs10877012 on 25(OH)D level has been verified in candidate gene studies, data regarding how this SNP regulates serum 25(OH)D is limited. Our study suggests that rs10877012 (CYP27B1) does alter the response of diabetic patients to vitamin D_3 supplementation. These results indicated that different mechanisms regulate 25(OH)D levels from vitamin D_3 supplementation. CYP27B1 functions downstream of circulating 25(OH)D; therefore, rs10877012 may alter the role of CYP27B1 in the metabolic feedback loop or regulate the metabolic rate of 25(OH)D [26]. The gene variant may reduce the efficiency of 25(OH) D hydroxylation to 1,25-dihydroxy vitamin D (1,25-(OH)2D), which may lead to a phenomenon that if the condition of 25(OH)D level is normal or even higher, $1,25(OH)_2D_3$ level in serum is low. In other words, some people may have mild "vitamin D resistance" but lack of the rickets displays.

The present study was a population-based and prospective study. The internal effectiveness was maximized by using uniform regimen and dosage of vitamin D_3 supplementation, and minimizing participant's variation. Serum 25(OH)D was measured every 6 months to eliminate seasonal effect and increase reproducibility. We used rigorous statistical methods to examine the interaction between genotype and vitamin D_3 supplementation, minimizing the effect of confounding factors on the results. Finally, we focused on candidate SNPs which are previously reported to be associated with 25(OH)D or other health outcomes, covering five key genes involved in the vitamin D metabolism. However, some limitations of the study are also worth mentioning. First, the relatively small sample size of our study may affect the results. It is necessary to expand the sample size to conduct more comprehensive analyses. Second, we did not examine the "downstream" markers for vitamin D status because 25(OH)D concentration is considered the most reliable indicator of vitamin D status. Other molecules, such as 1,25-(OH)2D or parathyroid hormone, have greater intraindividual variability and are affected by

other factors in addition to vitamin D status. Finally, we only studied diabetic patients in the Chinese population. It is unclear whether the genetic variations found in this study affect the vitamin D status in other ethnic populations or people with other diseases, which deserves further study.

5. Conclusions

In conclusion, we systematically studied the association between single nucleotide polymorphisms of vitamin D metabolism-associated genes and the change of serum 25(OH)D level after long-term vitamin D_3 supplementation in patients with type 2 diabetes. We found that CYP27B1 variation might be a predictive factor for vitamin D insufficiency after vitamin D_3 supplementation. Our study suggests that SNP analysis can identify high-risk diabetic patients who may require higher doses of vitamin D_3 supplementation, and vitamin D_3 supplementation should be personalized.

Data Availability

The data used to support the findings of this study are currently under embargo while the research findings are commercialized. Requests for data, 12 months after publication of this article, will be considered by the corresponding author.

Ethical Approval

All procedures performed in this study which involves human participants were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of Soochow University (ESCU-20160001) and retrospectively registered in the Chinese Clinical Trial Registry.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

ZYH and SST contributed equally as first authors and were responsible for collection and compilation of data and in writing the manuscript. HQL performed the data analysis. GTP and BYL contributed in the collection and compilation of the clinical data. ZLZ designed and supervised the study and revised the manuscript. All authors read and approved the final manuscript.

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Supplementary Materials

Table S1: the description of candidate SNPs genotyped. Table S2: a description of baseline 25(OH)D levels in patients with different SNP genotypes, which the RRs for low level of 25(OH)D associated with various SNPs was re-estimated using log-binomial regression. Table S3: a description of 25(OH)D level and insufficiency risk in patients with different CYP27B1 (10877012) genotypes after stratification of vitamin D metabolism-related indicators (sex, BMI, and TC), which the RRs for low level of 25(OH)D associated with various SNPs were re-estimated using log-binomial regression. *(Supplementary Materials)*

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Research Article

Effect of Early and Late Interventions with Dietary Oils on Vascular and Neural Complications in a Type 2 Diabetic Rat Model

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Aims. Determine the effect of dietary oils enriched in different mono- or polyunsaturated fatty acids, i.e., olive oil (18 : 1, oleic acid), safflower oil (18 : 2 n-6, linoleic acid), flaxseed oil (18 : 3 n-3, alpha linolenic acid), evening primrose oil (18 : 3 n-6, gamma linolenic acid), or menhaden oil (20:5/22 : 6 n-3 eicosapentaenoic/docosahexaenoic acids), on vascular and neural complications in high-fat-fed low-dose streptozotocin-treated Sprague-Dawley rats, an animal model for late-stage type 2 diabetes. *Materials and Methods*. Rats were fed a high-fat diet (45% kcal as fat primarily derived from lard) for 8 weeks and then treated with a low dose of streptozotocin (30 mg/kg) in order to induce hyperglycemia. After an additional 8 (early intervention) or 20 (late intervention) weeks, the different groups of rats were fed diets with 1/2 of the kcal of fat derived from lard replaced by the different dietary oils. In addition, a control group fed a standard diet (4.25% kcal as fat) and a diabetic group maintained on the high-fat diet were maintained. The treatment period was approximately 16 weeks. The endpoints evaluated included vascular reactivity of epineurial arterioles, motor and sensory nerve conduction velocity, thermal and corneal sensitivity, and innervation of sensory nerves in the cornea and skin. *Results*. Our findings show that menhaden and flaxseed oil provided the greatest benefit for correcting peripheral nerve damage caused by diabetes, whereas enriching the high-fat diet with menhaden oil provided the most benefit to acetylcholine-mediated vascular relaxation of epineurial arterioles of the sciatic nerve. Enriching the diets with fatty acids derived from the other oils provided none to partial improvements. *Conclusions*. These studies imply that long-chain n-6 and n-3 polyunsaturated fatty acids could be an effective treatment for diabetic peripheral neuropathy with n-3 polyunsaturated fatty acids derived from fish oil being the most effective.

1. Introduction

Peripheral neuropathy is the most common complication of diabetes affecting about 50% of patients [1]. There is no treatment for peripheral neuropathy other than good glycemic control that is primarily only effective in patients with type 1 diabetes [1]. Since symptoms of peripheral neuropathy include pain, numbness, paresthesia, and ulcerations in the extremities that can lead to amputations, finding a treatment to improve the quality of life is vital.

Lipids have been shown to play an important role in complications associated with metabolic syndrome and type 2 diabetes [1, 2]. Recently, in a similar study, we have shown that treating diet-induced obese rats, an animal model of pre-diabetes, after vascular and nerve pathology had developed, with oils enriched in oleic acid or linoleic acid provided no

improvement in vascular or neural function. In that study, we also found that treating diet-induced obese rats with either γ- or α-linolenic acids, derived from evening primrose oil or flaxseed oil, respectively, provided a moderate benefit. However, we found that treating diet-induced obese rats with menhaden oil enriched in eicosapentaenoic and docosahexaenoic acids provided significant improvement in both vascular and neural functions [3]. The purpose of the present study was to gain a better understanding of the role of these same dietary lipids on vascular relaxation of arterioles that provide circulation to the sciatic nerve and peripheral neuropathy in an animal model of type 2 diabetes. Sprague-Dawley rats treated with a high-fat diet as done in our previous study do not become hyperglycemic [3]. Therefore, for this study, we used Sprague-Dawley rats that were fed a high-fat diet for 8 weeks followed by a low dose of streptozotocin [4]. This approach creates a rat model of late-stage type 2 diabetes [4]. After 8 or 20 weeks of nontreated hyperglycemia, these rats were fed a high-fat diet enriched in oleic acid (olive oil; 18 : 1, n-9), linoleic acid (safflower oil; 18 : 2, n-6), γ-linolenic acid (evening primrose oil; 18 : 3, n-6), α-linolenic acid (flaxseed oil; 18 : 3, n-3), or eicosapentaenoic/docosahexaenoic acids (menhaden oil; 20 : 5 and 22 : 6, n-3).

2. Materials and Methods

2.1. Animals, Diets, and Experimental Design. Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats 10-11 weeks of age were housed in a certified animal care facility, and food and water were provided ad libitum. All institutional and NIH guidelines for use of animals were followed. These studies were approved by the University of Iowa Animal Care and Use Committee (# 5071450). At 12 weeks of age, rats were divided into 7 groups. One group was designated as the control group and remained on the standard diet (Envigo Teklad, #7001, Madison, WI), which contained 25% protein, 4% fat, and 40% carbohydrate. The other 6 groups of rats were placed on a high-fat diet for 8 weeks (D12451; Research Diets, New Brunswick, NJ). The high-fat diet contained 24% protein, 24% fat, and 41% carbohydrate, and 45% of the kcal in this diet is derived from fat. The primary source of the increased fat content in the diet was lard. Afterwards, these rats were treated with a low dose of streptozotocin (30 mg/kg) to induce hyperglycemia [4]. Following 8 weeks (early intervention) or 20 weeks (late intervention) of untreated hyperglycemia, 5 of the 6 groups of diabetic rats were placed on high-fat diets with 1/2 kcal of fat derived from lard replaced with olive oil (D16030902), safflower oil (D16030903), flaxseed oil (D16030904), evening primrose oil (D16030905), or menhaden oil (D16021504). The remaining group of diabetic rats was maintained on the original high-fat diet (D12451) while the control rats remained on the standard diet for the duration of the study. These diets were prepared by Research Diets, and the treatment period was approximately 16 weeks. The olive, safflower, flaxseed, and menhaden oils were provided by Research Diets. Evening primrose oil was purchased from Starwest Botanicals Inc. (Sacramento, CA) and shipped to Research Diets for production of D16030905. The fatty acid composition of each of these diets is presented in Supplemental Table 1.

2.2. Endpoints Related to Nerve Function and Vascular Reactivity. As in all our studies, multiple of endpoints related to neural function were determined employing methodology common to our laboratory and detailed information can be found in these references [5–7]. Moreover, the endpoints examined in this study were the same as the endpoints evaluated in our study of the effect of dietary lipids on vascular and neural dysfunction in diet-induced obese rats [3]. The endpoints examined included thermal nociceptive latency of the hindpaws and cornea sensitivity in unanesthetized rats. Motor and sensory nerve conduction velocity and density of corneal nerves by corneal confocal microscopy were determined in rats anesthetized with sodium pentobarbital (50 mg/kg, i.p., Abbott Laboratories, North Chicago, IL). Following these procedures, the anesthetized rats were euthanized by exsanguination via cardiac puncture and tissues were harvested to determine intraepidermal nerve fiber density of the hindpaw, vascular reactivity of epineurial arterioles of the sciatic nerve to acetylcholine and calcitonin gene-related peptide, and liver steatosis as previously described.

2.3. Fatty Acid Composition. Fatty acid composition of the diets, serum, and the liver were measured after the lipid fraction was extracted using chloroform/methanol, followed by transesterification in 14% boron trifluoride in methanol and extraction of the fatty acid methyl esters into heptane. The fatty acids were then separated by gas-liquid chromatography [8]. Individual fatty acids peak as % of total fatty acids present were identified by comparison to known fatty acid standards.

2.4. Physiological Markers. Nonfasting blood glucose was determined with Aviva Accu-Chek strips. Serum was collected under nonfasting conditions for determining the levels of free fatty acid, triglyceride, and free cholesterol using commercial kits and provided instructions from Roche Diagnostics, Mannheim, Germany; Sigma Chemical Co., and St. Louis, MO; Bio Vision, Mountain View, CA, respectively. Serum was also used to determine thiobarbituric acid-reactive substances as previously described [7].

2.5. Data Analysis. Results are presented as mean ± SEM. Comparisons between the groups were conducted using one-way ANOVA and Bonferroni posttest comparison (Prism software; GraphPad, San Diego, CA). Concentration response curves for acetylcholine and calcitonin gene-related peptide were compared using a two-way repeated measures analysis of variance with an autoregressive covariance structure using proc mixed program of SAS data for area under the curve which was also determined for each concentration curve using Prism software and values included in the figure legends [5–7]. A p value of less than 0.05 was considered significant.

3. Results

3.1. Weight and Blood Glucose. Tables 1 and 2 provide data on the starting and final weights and nonfasting blood glucose levels of rats entered into the early (Table 1) or late (Table 2) intervention protocols. At the beginning of the two study protocols, all rats weighed the same. At the end of the early intervention protocol, diabetic rats treated with olive oil, evening primrose oil, or menhaden oil weighed significantly less than control rats. For the late intervention protocol, there was no significant difference in the final weight of the rats. In both protocols, nonfasting blood glucose levels were significantly increased in diabetic rats and were not influenced by the different lipid-enriched diets. The amount of diet consumed was examined in weekly intervals, and diabetic rats generally ate about 15% more chow than control rats, and the lipid composition of the high-fat diets did not significantly affect this pattern (data not shown).

3.2. Serum Lipid and Liver Steatosis. Serum free fatty acid levels were significantly elevated in high-fat diet-fed diabetic rats after 24 weeks of diabetes (Table 1) and to a greater extent after 36 weeks (Table 2). With early intervention, serum free fatty acid levels at the end of the study were not increased in diabetic rats receiving the high-fat diet enriched with safflower oil or flaxseed oil. However, in the late intervention group, serum free fatty acid levels were significantly increased in all diabetic rats independent of dietary oil enrichment at the end of the study. Serum triglyceride levels were increased in all diabetic rats at the end of the early or late intervention study with a greater increase occurring in rats with 36 weeks of hyperglycemia (Table 2). There was no significant effect of dietary oils on serum triglyceride levels in diabetic rats. Serum cholesterol levels were increased in high-fat-fed diabetic rats. Early intervention with a high-fat diet enriched with safflower oil or flaxseed oil lowered cholesterol levels. Late intervention of diabetic rats with a high-fat diet enriched with evening primrose oil or flaxseed oil lowered cholesterol levels. Serum thiobarbituric acid substances, a marker of oxidative stress, were significantly increased in all diabetic rats and not altered after changing the dietary lipid content of the high-fat diet.

Recent studies have demonstrated an association between nonalcoholic fatty liver disease and diabetic neuropathy [9, 10]. Liver steatosis was increased in high-fat-fed diabetic rats after 24 (Table 1) or 36 (Table 2) weeks of hyperglycemia. Enriching the diets of high-fat-fed diabetic rats with oils containing mono- or polyunsaturated fatty acids partially lowered the fatty acid content of the liver compared to untreated high-fat-fed diabetic rats, but in all cases, liver steatosis remained significantly increased compared to control rats.

3.3. Fatty Acid Composition of Serum and the Liver. Supplemental Tables 2 and 3 present data for the fatty acid composition of serum following early and late dietary interventions, respectively. In serum, the primary fatty acid enriched in each of the oils was generally increased compared to control and/or high-fat-fed diabetic rats. For the early intervention (Supplemental Table 2), the increase was significant for each of the primary fatty acids derived from each of the dietary oils, and for the late intervention (Supplemental Table 3), a significant increase in the primary fatty acid in serum was achieved with only flaxseed oil and menhaden oil.

Supplemental Tables 4 and 5 present data for the fatty acid composition of the liver following early and late interventions, respectively. The changes that occurred in the fatty acid composition of the liver were similar to that observed in serum, and generally, there was an increase in the primary fatty acid derived from each of the dietary oils in the liver. The increase was only consistently significant for flaxseed oil and menhaden oil. Interestingly, the content of arachidonic acid was significantly decreased in the liver of diabetic rats treated with high-fat diets enriched in flaxseed oil or menhaden oil compared to control or untreated diabetic rats. Following the early intervention, the fatty acid unsaturation index was significantly increased in serum and liver of diabetic rats treated with a high-fat diet enriched in menhaden oil (Supplemental Table 6). In the late intervention study, the fatty acid unsaturation index was significantly increased in serum of diabetic rats treated with a high-fat diet enriched in menhaden oil and in the liver of diabetic rats treated with a high-fat diet enriched in either flaxseed oil or menhaden oil (Supplemental Table 7).

Recent studies have suggested that reducing the n-6 to n-3 fatty acid ratio improves inflammatory conditions [11, 12]. In this study, we found that treating high-fat-fed diabetic rats following an early or late intervention with a high-fat diet enriched in menhaden oil or to a lesser extent flaxseed oil significantly reduces the n-6 to n-3 fatty acid ratio in serum and the liver (Supplemental Table 8 or 9, respectively).

3.4. Neural and Vascular Endpoints. Data in Figures 1 and 2 demonstrate that motor and sensory nerve conduction velocity is decreased in Sprague-Dawley rats modeling type 2 diabetes with chronic hyperglycemia. Early intervention with evening primrose oil of the high-fat diet improved both motor and sensory nerve conduction velocities, but this treatment was ineffective in the late intervention protocol. Enriching the high-fat diet with flaxseed oil was also partially effective in improving motor and to a greater extent sensory nerve conduction velocity. However, enriching the high-fat diet early or late with menhaden oil significantly improved both motor and sensory nerve conduction velocities. Enriching the diet of diabetic rats with olive oil or safflower oil provided no improvement in either motor or sensory nerve conduction velocity whether it was given early or late after the onset of hyperglycemia.

Thermal nociception and density of sensory nerve fibers in the skin of the hindpaw are common neuro-related endpoints examined in preclinical studies of diabetes in rodents. Data in Figures 3 and 4 demonstrate that in a rat modeling type 2 diabetes, there is a significant decrease in nerve fibers in the skin and latent response to a thermal stimulus. Treating diabetic rats early or late with olive oil or safflower oil had minimal to no effect on these two endpoints. Treatment (late intervention) with evening primrose oil partially improved

Table 1: Effect of dietary oils on weight gain, serum lipids, and thiobarbituric acid substances and liver steatosis in type 2 diabetic Sprague-Dawley rats: early intervention.

Determination	Control (12)	Diabetic (14)	Diabetic+olive oil (12)	Diabetic+safflower oil (13)	Diabetic+evening primrose oil (12)	Diabetic+flaxseed oil (13)	Diabetic+menhaden oil (14)
Start weight (g)	362 ± 7	348 ± 5	352 ± 4	349 ± 4	349 ± 4	341 ± 3	349 ± 5
End weight (g)	538 ± 18	452 ± 22	459 ± 17[a]	479 ± 17	453 ± 12[a]	470 ± 18	454 ± 12[a]
Blood glucose (mg/dl)	138 ± 7	404 ± 27[a]	463 ± 28[a]	423 ± 28[a]	437 ± 26[a]	464 ± 25[a]	486 ± 20[a]
Serum free fatty acid (mmol/l)	0.09 ± 0.01	0.22 ± 0.04[a]	0.28 ± 0.04[a]	0.14 ± 0.03	0.48 ± 0.07[a,b]	0.15 ± 0.01	0.33 ± 0.02[a]
Serum triglycerides (mg/dl)	19 ± 3	183 ± 29[a]	281 ± 96[a]	198 ± 65[a]	238 ± 57[a]	130 ± 31[a]	176 ± 53[a]
Serum cholesterol (mg/ml)	4.2 ± 0.2	9.7 ± 0.9[a]	13.5 ± 2.2[a]	7.6 ± 0.7	8.6 ± 1.0[a]	7.4 ± 0.6	9.6 ± 1.2[a]
Serum thiobarbituric acid substances (μg/ml)	0.55 ± 0.08	1.15 ± 0.11[a]	1.05 ± 0.07[a]	1.02 ± 0.10[a]	1.02 ± 0.08[a]	1.15 ± 0.14[a]	1.15 ± 0.14[a]
Liver steatosis (%)	5.1 ± 0.5	40.8 ± 1.3[a]	33.2 ± 1.2[a]	30.8 ± 1.5[a,b]	34.0 ± 1.5[a]	30.1 ± 1.9[a,b]	29.3 ± 1.4[a,b]

Data are presented as the mean ± S.E.M. [a]$P < 0.05$ compared to control rats. [b]$P < 0.05$ compared to diabetic rats. Parentheses indicate the number of experimental animals.

Table 2: Effect of dietary oils on weight gain, serum lipids, and thiobarbituric acid substances and liver steatosis in type 2 diabetic Sprague-Dawley rats: late intervention.

Determination	Control (10)	Diabetic (12)	Diabetic+olive oil (12)	Diabetic+safflower oil (12)	Diabetic+evening primrose oil (12)	Diabetic+flaxseed oil (12)	Diabetic+menhaden oil (12)
Start weight (g)	346 ± 5	358 ± 3	347 ± 6	352 ± 4	349 ± 3	340 ± 6	352 ± 6
End weight (g)	547 ± 21	496 ± 19	506 ± 21	496 ± 15	498 ± 21	514 ± 21	520 ± 20
Blood glucose (mg/dl)	126 ± 4	438 ± 48[a]	431 ± 46[a]	389 ± 46[a]	447 ± 46[a]	361 ± 47[a]	424 ± 48[a]
Serum free fatty acid (mmol/l)	0.13 ± 0.01	0.44 ± 0.08[a]	0.52 ± 0.04[a]	0.54 ± 0.14[a]	0.36 ± 0.08[a]	0.41 ± 0.05[a]	0.60 ± 0.07[a]
Serum triglycerides (mg/dl)	47 ± 12	773 ± 176[a]	1248 ± 270[a]	613 ± 191[a]	394 ± 49[a]	536 ± 134[a]	829 ± 156[a]
Serum cholesterol (mg/ml)	4.7 ± 0.5	9.4 ± 1.3[a]	8.9 ± 1.5[a]	12.6 ± 1.5[a]	7.6 ± 1.0	7.4 ± 0.6	8.6 ± 0.9[a]
Serum thiobarbituric acid substances (μg/ml)	0.42 ± 0.04	1.20 ± 0.13[a]	1.15 ± 0.09[a]	1.13 ± 0.13[a]	1.12 ± 0.08[a]	1.25 ± 0.13[a]	1.25 ± 0.14[a]
Liver steatosis (%)	5.8 ± 0.4	44.4 ± 1.2[a]	36.2 ± 1.2[a,b]	34.1 ± 1.1[a,b]	35.4 ± 1.2[a,b]	33.5 ± 1.5[a,b]	31.8 ± 1.4[a,b]

Data are presented as the mean ± SEM. [a]$P < 0.05$ compared to control rats. [b]$P < 0.05$ compared to diabetic rats. Parentheses indicate the number of experimental animals.

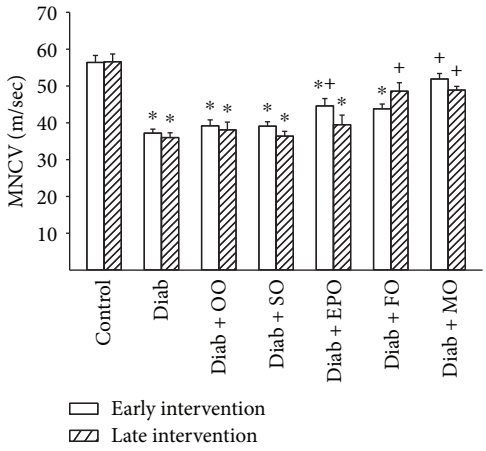

Figure 1: Effect of dietary oils on motor nerve conduction velocity in high-fat-fed diabetic Sprague-Dawley rats. Motor nerve conduction velocity was examined after early (open bars) and late (hatched bars) interventions as described in Materials and Methods. Data are presented as the mean ± SEM in m/sec. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Motor nerve conduction velocities for control and diabetic rats at the beginning of the early intervention treatment were 52.5 ± 1.7 and 37.4 ± 1.7 m/sec, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

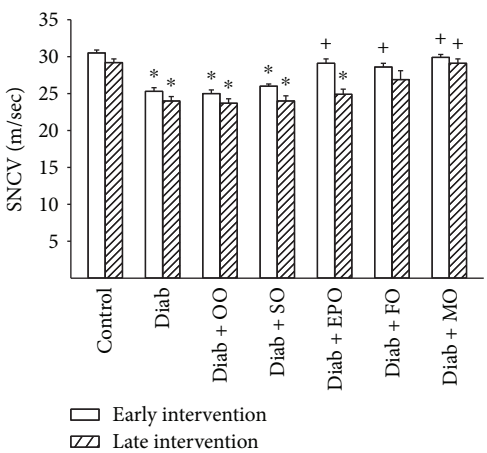

Figure 2: Effect of dietary oils on sensory nerve conduction velocity in high-fat-fed diabetic Sprague-Dawley rats. Sensory nerve conduction velocity was examined after early (open bars) and late (hatched bars) interventions as described in Materials and Methods. Data are presented as the mean ± SEM in m/sec. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Sensory nerve conduction velocities for control and diabetic rats at the beginning of the early intervention treatment were 31.9 ± 1.1 and 26.9 ± 0.7 m/sec, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

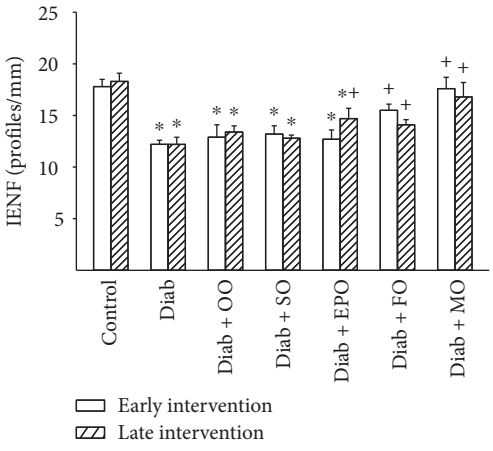

Figure 3: Effect of dietary oils on intraepidermal nerve fiber density in high-fat-fed diabetic Sprague-Dawley rats. Intraepidermal nerve fiber density was examined after early (open bars) and late (hatched bars) interventions as described in Materials and Methods. Data are presented as the mean ± SEM in profiles per mm. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Intraepidermal nerve fiber densities for control and diabetic rats at the beginning of the early intervention treatment were 16.9 ± 1.0 and 13.1 ± 0.6 profiles/mm, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

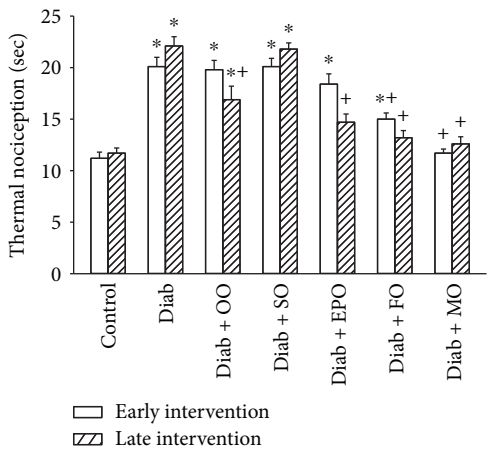

Figure 4: Effect of dietary oils on thermal nociception in high-fat-fed diabetic Sprague-Dawley rats. Thermal nociception was examined after early (open bars) and late (hatched bars) interventions as described in Materials and Methods. Data are presented as the mean ± SEM in sec. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Thermal nociception for control and that for diabetic rats at the beginning of the early intervention treatment were 12.3 ± 0.2 and 20.1 ± 0.9 sec, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

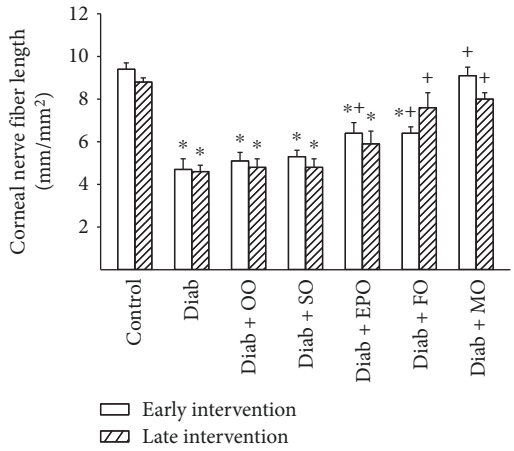

Figure 5: Effect of dietary oils on cornea nerve fiber length in high-fat-fed diabetic Sprague-Dawley rats. Innervation of the subepithelial layer of the cornea was determined after early (open bars) and late (hatched bars) interventions by using corneal confocal microscopy as described in Materials and Methods. Data are presented as the mean ± SEM for innervation of the cornea in mm/mm^2. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Cornea nerve fiber lengths for control and diabetic rats at the beginning of the early intervention treatment were 8.4 ± 0.4 and $4.4 \pm 0.5\,mm/mm^2$, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

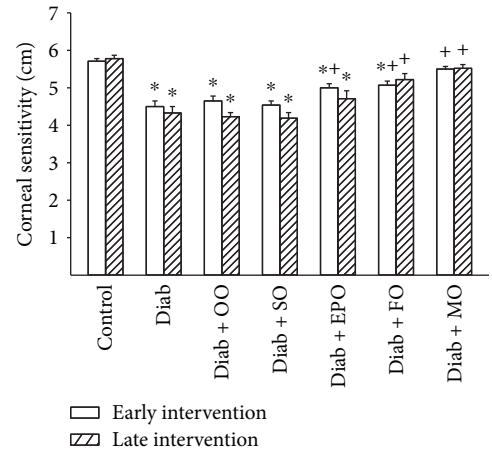

Figure 6: Effect of dietary oils on cornea sensitivity in high-fat-fed diabetic Sprague-Dawley rats. Corneal sensitivity was determined after early (open bars) and late (hatched bars) interventions by using Cochet-Bonnet filament esthesiometer as described in Materials and Methods. Data are presented as the mean ± SEM for corneal sensitivity in cm. The number of rats in each group was the same as shown in Tables 1 and 2, for the early and late intervention periods, respectively. Corneal sensitivities for control and diabetic rats at the beginning of the early intervention treatment were 5.9 ± 0.1 and 5.0 ± 0.2 cm, respectively. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to diabetic rats. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

the density of intraepidermal nerve fibers and thermal nociception. Treating diabetic rats fed a high-fat diet by replacing 1/2 of the kcal of the high-fat diet with flaxseed oil or to a greater extent menhaden oil significantly improved both intraepidermal nerve fiber density and thermal nociception.

Recently, examination of corneal nerve fiber density of the subepithelial layer and cornea sensitivity has been promoted as a surrogate marker for progression of diabetic neuropathy in human subjects [13]. Data in Figures 5 and 6 demonstrate that chronic hyperglycemia causes a decrease in corneal nerves and abnormal cornea sensitivity. Treating high-fat-fed diabetic rats early or late after the onset of hyperglycemia with high-fat diets enriched with olive oil or safflower oil did not correct cornea nerve fiber density or corneal sensitivity. Early intervention with evening primrose oil was partially effective in improving cornea nerve fiber density and sensitivity. Early and late interventions with flaxseed oil were also partially effective. However, early or late dietary intervention of diabetic rats with menhaden oil completely restored both cornea nerve density and sensitivity.

We have previously demonstrated that vasodilation of epineurial arterioles, blood vessels that provide circulation to the sciatic nerve, to acetylcholine is decreased prior to slowing of nerve conduction velocity in diabetic rats, and after 8 weeks of chronic hyperglycemia vasodilation to calcitonin, gene-related peptide is also decreased [14, 15]. At the time of early intervention, vascular relaxation of epineurial arterioles to acetylcholine and calcitonin gene-related peptide was significantly decreased (Supplemental Figure 1). Data in Figures 7 and 8 confirm that acetylcholine-mediated vascular relaxation is impaired in rats modeling type 2 diabetes after 24 (Figure 7) or 36 (Figure 8) weeks of chronic and untreated hyperglycemia. Treating high-fat-fed diabetic rats by enriching their diets with olive oil or safflower oil was the least effective in improving vascular reactivity to acetylcholine regardless of early or late intervention. Treating diabetic rats by dietary enrichment with either evening primrose oil or flaxseed oil was partially effective in improving vascular relaxation to acetylcholine. However, the greatest benefit to acetylcholine-mediated vascular relaxation was observed when diabetic rats were treated early or late with a diet enriched with menhaden oil. Data in Figures 9 and 10 demonstrate that chronic hyperglycemia for 24 (Figure 9) or 36 weeks (Figure 10) causes a significant decrease in vascular relaxation of epineurial arterioles to calcitonin gene-related peptide. Early intervention by replacing 1/2 of the kcal of fat in the high-fat diet with olive oil, safflower oil, evening primrose oil, or flaxseed oil provided no observable improvement in vascular relaxation by diabetic rats to calcitonin gene-related peptide (Figure 9). However, with early intervention, improvement in vascular relaxation to calcitonin gene-related peptide was observed when the diet of high-fat-fed diabetic rats was enriched with menhaden oil. Late intervention of high-fat-fed diabetic rats with diets enriched with menhaden oil as well as with evening primrose oil or flaxseed oil provided partial improvement in vascular relaxation to calcitonin gene-

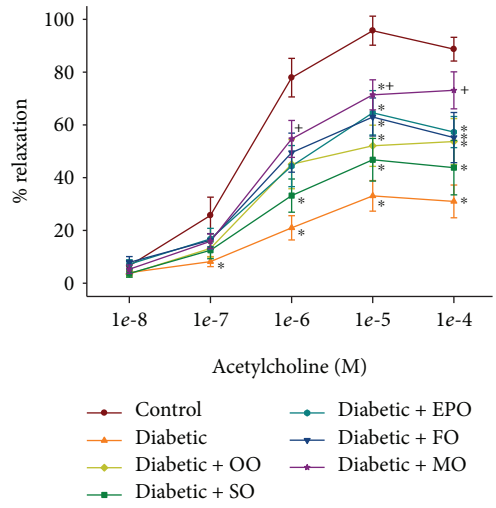

Figure 7: Effect of early intervention with dietary oils on vascular relaxation by acetylcholine in epineurial arterioles of the sciatic nerve in high-fat-fed diabetic Sprague-Dawley rats. Pressurized arterioles (40 mm Hg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30-50%), and incremental doses of acetylcholine were added to the bathing solution while recording the steady-state vessel diameter. Data are presented as the mean of % relaxation ± SEM. The number of rats in each group was the same as shown in Table 1. Using area under the curve to compare the effect of diabetes and treatments to relaxation by acetylcholine: control vs. diabetic, $P < 0.001$; control vs. diabetic+OO, $P < 0.02$; control vs. diabetic+SO, $P < 0.001$; control vs. diabetic+EPO, $P < 0.005$; control vs. diabetic+FO, $P < 0.05$; diabetic vs. diabetic+MO, $P < 0.02$. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

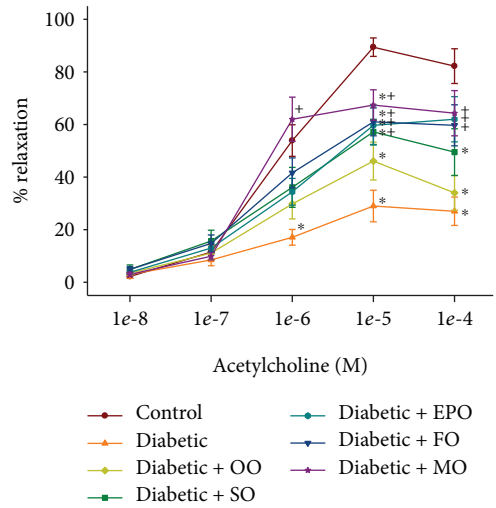

Figure 8: Effect of late intervention with dietary oils on vascular relaxation by acetylcholine in epineurial arterioles of the sciatic nerve in high-fat-fed diabetic Sprague-Dawley rats. Pressurized arterioles (40 mm Hg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30-50%), and incremental doses of acetylcholine were added to the bathing solution while recording the steady-state vessel diameter. Data are presented as the mean of % relaxation ± SEM. The number of rats in each group was the same as shown in Table 2. Using area under the curve to compare the effect of diabetes and treatments to relaxation by acetylcholine: control vs. diabetic, $P < 0.001$; control vs. diabetic+OO, $P < 0.01$; diabetic vs. diabetic+FO, $P < 0.05$; diabetic vs. diabetic+MO, $P < 0.005$. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

related peptide (Figure 10). In contrast, late intervention of diabetic rats with a diet enriched with olive oil or safflower oil provided no improvement in vascular relaxation to calcitonin gene-related peptide.

4. Discussion

Limited information is available on the effect that mono- or polyunsaturated fatty acids of the omega-6 or omega-3 classes may have on vascular and neural defects on rats modeling late-stage type 2 diabetes. To address this question, we investigated the effect of 16-week treatment of high-fat-fed diabetic rats following early (8 weeks post hyperglycemia) or late (20 weeks post hyperglycemia) intervention of hyperglycemia/high-fat diet with 1/2 of the kcal of fat derived from lard replaced with olive oil (enriched in oleic acid, 18 : 1 n-9 mono-unsaturated fatty acid), safflower oil (enriched in linoleic acid, 18 : 2 n-6 polyunsaturated fatty acid), evening primrose oil (enriched in γ-linolenic acid, 18 : 3 n-6 polyunsaturated fatty acid), flaxseed oil (enriched in α-linolenic acid, 18 : 3 n-3 polyunsaturated fatty acid), or menhaden oil (enriched in eicosapentaenoic and docosahexaenoic fatty acid, 20 : 5 and 22 : 6 n-3 polyunsaturated fatty acid). At the time of the early intervention treatment, diabetic rats had impaired neural function as determined by slowing of motor and sensory nerve conduction velocity, decreased density of intraepidermal nerve fibers and corneal nerve fiber length, and decreased thermal nociception and cornea sensitivity. In addition, at the time of early intervention, vascular relaxation by epineurial arterioles to acetylcholine and calcitonin gene-related peptide was decreased. At the time of late intervention, vascular and neuropathy endpoints were not significantly more impaired than at the time of early intervention. This is consistent with our earlier longitudinal studies that have shown a progressive decline in vascular and neural functions from 2 to 8 weeks post hyperglycemia after which the pathology in this late-stage type 2 diabetic rat model stabilizes. Nonetheless, we deemed it important to examine both early and late phases of diabetes to determine whether vascular and neural endpoints are reversible after prolonged hyperglycemia.

Treating diabetic rats with a high-fat diet enriched with oleic acid (olive oil) except for some improvement in thermal nociception with late intervention had no significant benefit on the neural endpoints and vascular relaxation to acetylcholine, and calcitonin gene-related peptide remained significantly impaired. We obtained similar results when we enriched diets of diet-induced obese rats with olive oil [3]. The serum lipid profile after early and late interventions was not changed compared to high-fat-fed diabetic rats nor was the fatty acid unsaturation index or n-6 to n-3 fatty acid ratio of serum or the liver. In addition, liver steatosis and a serum marker of oxidative stress remained elevated in

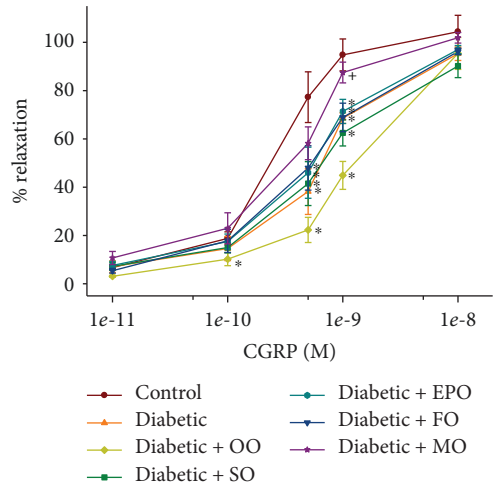

Figure 9: Effect of early intervention with dietary oils on vascular relaxation by calcitonin gene-related peptide in epineurial arterioles of the sciatic nerve in high-fat-fed diabetic Sprague-Dawley rats. Pressurized arterioles (40 mm Hg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30-50%), and incremental doses of calcitonin gene-related peptide were added to the bathing solution while recording the steady-state vessel diameter. Data are presented as the mean of % relaxation ± SEM. The number of rats in each group was the same as shown in Table 1. Using area under the curve to compare the effect of diabetes and treatments to relaxation by calcitonin gene-related peptide: control vs. diabetic, $P < 0.05$; control vs. diabetic+OO, $P < 0.01$; control vs. diabetic+SO, $P < 0.05$; control vs. diabetic+EPO, $P < 0.05$; control vs. diabetic+FO, $P < 0.05$; diabetic vs. diabetic+MO, $P < 0.05$. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

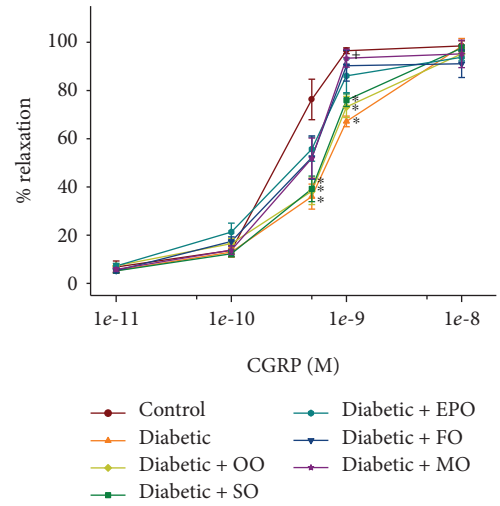

Figure 10: Effect of late intervention with dietary oils on vascular relaxation by calcitonin gene-related peptide in epineurial arterioles of the sciatic nerve in high-fat-fed diabetic Sprague-Dawley rats. Pressurized arterioles (40 mm Hg and ranging from 60 to 100 μm luminal diameters) were constricted with phenylephrine (30-50%), and incremental doses of calcitonin gene-related peptide were added to the bathing solution while recording the steady-state vessel diameter. Data are presented as the mean of % relaxation ± SEM. The number of rats in each group was the same as shown in Table 2. Using area under the curve to compare the effect of diabetes and treatments to relaxation by calcitonin gene-related peptide: control vs. diabetic, $P < 0.05$; control vs. diabetic+OO, $P < 0.05$; control vs. diabetic+SO, $P < 0.05$; diabetic vs. diabetic+MO, $P < 0.05$. OO: olive oil; SO: safflower oil; EPO: evening primrose oil; FO: flaxseed oil; MO: menhaden oil.

diabetic rats treated with olive oil. Nutritional intake of olive oil is the key component of the Mediterranean diet that has been associated with the prevention and management of many chronic diseases including type 2 diabetes [16, 17]. However, the Mediterranean diet is rich in extra virgin olive oil that is enriched with vitamins and polyphenols that have been shown to have antioxidative and anti-inflammatory properties [18–20]. Extra virgin olive oil has been shown to ameliorate nonalcoholic steatohepatitis in high-fat Western diet-treated mice [21]. It is believed that reducing saturated fat intake through increasing consumption of monounsaturated fatty acids or polyunsaturated fatty acids will reduce obesity and conditions associated with metabolic syndrome [22]. In our studies, replacing 1/2 of the kcal derived from saturated fatty acids from lard with olive oil was found to be beneficial in reducing hepatic steatosis and thermal nociception after late intervention; however, improvements appeared marginal and likely not of physiological significance. This lack of effect could be due to the conditions of the study design that even with early intervention, the olive oil-enriched diet was not able to overcome the existing deleterious effects of the high-fat diet/hyperglycemia condition on vascular and neural endpoints prior to treatment and/or the olive oil used in the diet did not have a sufficient concentration of vitamins and polyphenols of extra virgin olive oil.

The rationale for treating diabetes complications including vascular dysfunction and neuropathy with polyunsaturated fatty acids such as gamma linolenic acid is because of the diabetes-induced impairment of delta-6-desaturase [23–25]. Delta-6-desaturase converts linoleic acid into gamma linolenic acid and it is thought that increasing the intake of gamma linolenic acid in diabetes would circumvent this issue. In our studies, treating diabetic rats with a high-fat diet with 1/2 of the kcal derived from safflower oil that is enriched in linoleic acid (18 : 2, n-6 polyunsaturated fatty acid) did not improve vascular or neural endpoints. This is likely due to the inability to convert linoleic acid to gamma linolenic acid. Levels of linoleic acid were increased in the serum and the liver of safflower oil-treated rats, but there was no increase in gamma linolenic acid. Evening primrose oil is enriched in gamma linolenic acid (18 : 3, n-6 polyunsaturated fatty acid). In this study, treating type 2 diabetic rats with a high-fat diet enriched with evening primrose oil partially improved vascular and neural functions. We obtained similar results when diabetic rats were treated with flaxseed oil, which is enriched in alpha linolenic acid (18 : 3, n-3 polyunsaturated fatty acid). The levels of gamma linolenic acid in serum and the liver where only marginally but nonsignificantly increased when diabetic rats were treated with evening primrose oil except for the increase in serum following early intervention

(Supplemental Table 2). This result is of interest because treating diabetic rats with evening primrose oil partially improved motor and sensory nerve conduction only with early intervention. It is unknown whether the small increase in serum levels of gamma linolenic acid with early intervention contributed to the marginal increase in nerve conduction velocities in diabetic rats treated with evening primrose oil. The unsaturation index of serum or the liver was not increased in diabetic rats treated with gamma linolenic acid nor was the n-6 to n-3 fatty acid ratio suggesting that these parameters were not predictive of the small improvement in nerve conduction velocities with early intervention of evening primrose oil of diabetic rats. In contrast, levels of alpha linolenic acid were significantly increased in serum and the liver of diabetic rats when treated with flaxseed oil. This is likely because levels of gamma linolenic acid in evening primrose oil compared to levels of alpha linolenic acid in flaxseed oil are much lower (Supplemental Table 1). Nonetheless, evening primrose oil has been widely used as a source of gamma linolenic acid in preclinical studies. In early studies, evening primrose oil was shown to improve nerve conduction velocity in streptozotocin type 1 diabetic rats presumably by improving sciatic nerve blood flow and endoneurial oxygen tension [26, 27]. Treating streptozotocin type 1 diabetic rats with evening primrose oil has also been shown to improve ultrastructural deficits of axons as well as axonal transport and restore Na+/K+ ATPase activity in the sciatic nerve [23, 28, 29]. To the best of our knowledge, no preclinical studies have been performed examining the effect of flaxseed oil on diabetic peripheral neuropathy. However, it has been shown that dietary flaxseed oil ameliorates renal oxidative stress, protein glycation, and inflammation in streptozotocin-nicotinamide-induced diabetic rats, a model for type 2 diabetes [30]. The authors of this study suggested that n-3 polyunsaturated fatty acids may slow progression of diabetic nephropathy. Flaxseed oil has also been shown to attenuate hepatic steatosis and insulin resistance in high-fat-fed mice through improving endoplasmic reticulum stress [31]. In type 2 diabetic rats, flaxseed oil has been shown to alleviate protein glycation and inflammation in the liver [32, 33]. In our studies, we found that treating diabetic rats with flaxseed oil lead to an increase in the levels of eicosapentaenoic acid in serum and liver and a significant decrease in the n-6 to n-3 fatty acid ratio in serum. Overall, there was a trend for late intervention with flaxseed oil to be more efficacious in improving neural outcome measures and vascular relaxation to calcitonin gene-related peptide compared to early intervention. This was surprising since the duration of treatment was the same for both early and late interventions. It is possible that prolonged hyperglycemia may lead to increased sensitivity to exposure of long-chain n-3 polyunsaturated fatty acids. Long-chain n-3 polyunsaturated fatty acids such as eicosapentaenoic acid have been shown to have anti-inflammatory properties. Interestingly, analysis of data from the National Health and Nutrition Examination Survey found that adults with diabetes whose linolenic acid intake was in the highest quintile had lower odds of peripheral neuropathy than adults in the lowest quintile [34]. This analysis is supported by a small clinical double-blind placebo-controlled study done over 25 years ago that demonstrated that treating diabetic patients with distal diabetic polyneuropathy with 360 mg of gamma linolenic acid for 6 months provided significant improvement in neuropathy-related endpoints [35]. Future studies need to carefully evaluate the effect of treatment of diabetes on animal models as well as on human subjects with n-6 and n-3 polyunsaturated fatty acids on lipidomics and inflammatory mediators.

In treating type 2 diabetic rats with mono- or polyunsaturated fatty acids, the greatest benefit toward improving vascular dysfunction and neuronal activity in diabetic rats was observed with menhaden oil that is enriched with eicosapentaenoic and docosahexaenoic acids (20 : 5 and 22 : 6, respectively, n-3 polyunsaturated fatty acids). Diabetic rats treated with menhaden oil had a significant increase in both of these fatty acids in serum and the liver. There was also a significant increase in the unsaturation index in serum and the liver and a significant decrease in the ratio of n-6 to n-3 fatty acids in serum and the liver. Reducing the n-6 to n-3 fatty acid ratio is associated with a decrease in inflammatory stress [11]. The n-3 polyunsaturated fatty acids, eicosapentaenoic and docosahexaenoic acids, are precursors for E and D class resolvins that are credited for the anti-inflammatory properties of these fatty acids [36, 37]. Previously, we have demonstrated that treating streptozotocin diabetic mice with daily injections of resolvins improved neural endpoints and also stimulated nerve filament elongation in vitro [38, 39].

5. Conclusions

Our studies have shown that treating a rat model of type 2 diabetes with long-chain n-6 or n-3 polyunsaturated fatty acids following an early or late intervention protocol can improve vascular and neural deficits. Treating diabetic rats with either gamma or alpha linolenic acids, derived from evening primrose oil or flaxseed oil, respectively, provided a moderate benefit. In contrast, treating these rats with menhaden oil enriched in eicosapentaenoic and docosahexaenoic acids provided significant improvement in vascular and neural dysfunction. We previously reported similar results after dietary lipid modification of diet-induced obese rats leading us to conclude that increasing the dietary intake of fish oils may be a potential treatment for vascular and neural complications associated with prediabetes or type 2 diabetes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The content of this manuscript is new and solely the responsibility of the authors and does not necessarily represent the official views of the granting agencies.

Conflicts of Interest

The authors of this paper have no conflict of interest to report.

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Supplementary Materials

Supplementary Material 1. Supplemental Table 1: fatty acid % composition of diets 2. Supplemental Table 2: effect of dietary lipids on fatty acid % composition of serum measured by gas-liquid chromatography following early intervention 3. Supplemental Table 3: effect of dietary lipids on fatty acid % composition of serum measured by gas-liquid chromatography following late intervention 4. Supplemental Table 4: effect of dietary Lipids on fatty acid % composition of the liver measured by gas-liquid chromatography following early intervention 5. Supplemental Table 5: effect of dietary lipids on fatty acid % composition of the liver measured by gas-liquid chromatography following late intervention 6. Supplemental Table 6: fatty acid unsaturation indices of serum and the liver following early intervention 7. Supplemental Table 7: fatty acid unsaturation indices of serum and the liver following late intervention 8. Supplemental Table 8: ratio of n-6 to n-3 fatty acids of the liver and serum measured by gas-liquid chromatography following early intervention 9. Supplemental Table 9: ratio of n-6 to n-3 fatty acids of the liver and serum measured by gas-liquid chromatography following late intervention 10. Supplemental Figure 1: vascular relaxation to acetylcholine (top) and calcitonin gene-related peptide (bottom) in epineurial arterioles of the sciatic nerve at the time of early intervention in high-fat-fed diabetic Sprague-Dawley rats. *(Supplementary Materials)*

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