

Circulating milk fat globule-epidermal growth factor 8 levels are increased in pregnancy and gestational diabetes mellitus

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Keywords

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ABSTRACT

Aims/Introduction: Milk fat globule-epidermal growth factor 8 (MFG-E8) is the key mediator in anti-inflammatory responses that facilitate phagocytosis of apoptotic cells, and play an essential role in type 2 diabetes and pregnancy, both of which are under a low-grade inflammatory state. However, the action of MFG-E8 in gestational diabetes mellitus (GDM) is unclear. We measured plasma MFG-E8 levels in pregnancy and GDM for the first time, and elucidated possible relationships between its plasma levels and various metabolic parameters.

Materials and Methods: Plasma MFG-E8 levels were quantified by enzyme-linked immunosorbent assay in 66 women with GDM, 70 with normal pregnancy (p-NGT) and 44 healthy non-pregnant controls (CON), who were matched for age and body mass index. Inflammatory factors tumor necrosis factor- α (TNF- α) and C-reactive protein levels were measured, oral glucose tolerance test was carried out and β -cell function was evaluated.

Results: Plasma MFG-E8 levels were remarkably higher in p-NGT than in CON ($P = 0.024$), and were further elevated in GDM vs p-NGT ($P = 0.016$). MFG-E8 concentrations correlated positively with hemoglobin A1c, glucose levels and insulin resistance (homeostasis model assessment for insulin resistance), and correlated inversely with TNF- α and insulin secretion evaluated by disposition indices in pregnancies. Fasting glucose levels, disposition index of first phase insulin secretion and TNF- α were independent predictors of MFG-E8 levels in pregnancies. Logistic regression analyses showed that women in the third tertile of MFG-E8 levels had a markedly elevated risk of GDM.

Conclusions: Circulating MFG-E8 levels are dramatically elevated in pregnancy, and are significantly higher in GDM vs p-NGT. MFG-E8 concentrations are significantly associated with TNF- α , fasting glucose levels, homeostasis model assessment for insulin resistance and disposition indices. However, further studies are required to elucidate the regulation mechanism of MFG-E8 during pregnancy and GDM.

INTRODUCTION

In recent years, there have been increased debates regarding the pivotal role of inflammation in metabolic disorders. It is a widely shared belief that human pregnancy is featured by a

chronic, low-grade inflammatory state in comparison with the non-pregnant condition, which is mainly caused by the lack of physical exercise and a high calorie intake, leading to systemic insulin resistance. Gestational diabetes mellitus (GDM) occurs when the pancreatic β -cell reserve is inadequate to compensate for the enhanced insulin resistance with the onset or first recognition of pregnancy, and is reported in approximately 7% of all pregnant women according to the 2014 American Diabetes

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Association. Although GDM disappears after parturition, it is generally related to an enhanced risk of progression to obesity and type 2 diabetes mellitus in women. Correlations between insulin resistance and inflammatory factors, such as tumor necrosis factor- α (TNF- α), C-reactive protein (CRP) or interleukin-6 have been identified in GDM, even in normal pregnant women^{1,2}. Kirwan *et al.*³ reported that in both normal pregnancies and GDM, TNF- α was the most powerful predictor of insulin resistance during gestation, whereas estradiol, progesterone, human placental lactogen and other placenta-derived hormones were not considered specific markers of insulin resistance.

Milk fat globule-epidermal growth factor 8 (MFG-E8; also known as lactadherin in humans) was originally characterized as a secreted glycoprotein of the milk-fat globule, and was found to facilitate phagocytosis of apoptotic cells^{4,5}. MFG-E8 binds to apoptotic cells through the phosphatidylserine in the C-terminal, while it attaches to $\alpha_v\beta_3/\beta_5$ integrin expressed on activated macrophages through the arginine-glycine-aspartate motif of the N-terminal epidermal growth factor repeats. Two isoforms of MFG-E8 have been identified in murine mice, humans only have the short variant. The long form contains the proline/threonine-rich domain between the second epidermal growth factor-like repeat and the discoidin domain-1, which distinguishes it from the short variant. In mice, the long variant shows a limited tissue distribution, and is predominant during the late stages of gestation and during lactation; whereas the short form is expressed in various tissues, and substantially decreases in a lactation-dependent manner⁶. To date, MFG-E8 has been implicated in various human inflammatory diseases, and shows an anti-inflammation property in coronary atherosclerotic heart disease, chronic obstructive pulmonary disease, ulcerative colitis and rheumatoid arthritis^{7–10}, whereas inversely in brain ischemia¹¹ and atherosclerosis¹². Although known to be a mediator of inflammation, MFG-E8 is a multi-functional molecule that is involved in several cell surface-mediated regulatory events, such as tissue remodeling, tumor promotion, angiogenesis and facilitation of fertilization^{13–16}.

MFG-E8 and its receptor, $\alpha_v\beta_3/\beta_5$ integrin, are predominantly localized to the human endometrial epithelium. Based on a microarray analysis, Rehman *et al.*¹⁷ reported that MFG-E8 expression was 4.9-fold higher in the myometrium of pregnant women than in non-pregnant women, suggesting that MFG-E8 might play an essential role during human pregnancy. Bocca *et al.*¹⁸ confirmed that MFG-E8 functioned as a modulator of endometrial physiology and trophoblast adhesion and invasion by regulating the essential processes of remodeling, apoptosis, and angiogenesis in humans. Furthermore, MFG-E8 is an epithelial cell secretory product, and has been detected in microvesicles in milk and seminal plasma, as well as in the peripheral circulation¹⁹. Previous studies have shown a significant relationship between MFG-E8 and pregnancy in the uterus. However, there are no reports with data directly referring to MFG-E8 and pregnancy with or without normal

glucose tolerance in the human blood circulation. As MFG-E8 is a secreted protein, we examined plasma MFG-E8 levels using enzyme-linked immunosorbent assay to determine its clinical significance in pregnant women with or without diabetes. Pro-inflammatory cytokines originating from adipose tissue in obesity are a primary driver of inflammation; therefore, a state of adiposity before pregnancy is associated with the risk of pregnancy-related hypertension and diabetes²⁰. Women who were overweight had a body mass index (BMI) between 25 and 30 kg/m², and those with a BMI ≥ 30 kg/m² (regarded as obese, according to the 2004 World Health Organization criteria) before pregnancy were excluded from the current study to eliminate the interference of inflammation and insulin resistance.

MATERIALS AND METHODS

Study population

From June 2015 to April 2016, non-pregnant women were recruited from healthy volunteers, and gestational women were recruited from outpatients at the Department of Endocrinology and Metabolism and Department of Obstetrics and Gynecology at Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai, China. Informed consent was obtained from all participants, and the protocol was approved by the local ethics committee according to the Declaration of Helsinki as revised in 2008 before enrollment for the study. Women who met any of the following criteria were excluded from the study: family history of diabetes mellitus, multiple gestations, previous history of diabetes mellitus or other complications, hepatic disease, renal disease, thyroid dysfunction, hypertension, proteinuria, hematopathy or a pre-pregnancy BMI (pre-BMI; calculated as weight in kilograms divided by the square of height in meters) < 18.5 or > 25 . At 24–28 weeks of gestation, pregnant women were recruited at the Department of Gynecology and Obstetrics of Sixth People's Hospital, Shanghai, China, when they visited for a routine prenatal examination, and were subjected to a standardized 50-g glucose challenge test. Individuals with 1-h plasma glucose level ≥ 7.2 mmol/L were considered glucose challenge test positive, and returned within 1 week for blood tests and screening tests after overnight fasting. A 75-g oral glucose tolerance test (OGTT) was administered, and blood glucose and insulin levels at 0, 30, 60, 120, and 180 min were measured after glucose administration. GDM was diagnosed using the following threshold based on the American Diabetes Association 2013 guidelines: fasting plasma glucose levels ≥ 5.1 mmol/L and/or 60-min plasma glucose ≥ 10.0 mmol/L and/or 120 min plasma glucose levels ≥ 8.5 mmol/L.

Based on all these thresholds, 66 pregnant women were defined as having GDM, whereas 70 age- and pre-BMI-matched pregnancies were classified under normal glucose tolerance. Meanwhile, healthy, lean non-pregnant women with no previous history of pregnancy or diabetes were enrolled in the control group (CON). All controls underwent a 75-g OGTT to exclude impaired glucose tolerance.

Anthropometric and biochemical measurements

The screening tests included a physical examination and a review of the clinical history of the participants. On the day of the test, the height, weight, systolic blood pressure (SP), diastolic blood pressure (DP), and gestational days of each participant were assessed and recorded. BMI was determined as the weight (in kilograms) at blood collection divided by the square of the height (in meters). Plasma glucose was determined using the oxidase method with a Glamour 2000 Automatic Biochemical Analyzer (Molecular Devices, Sunnyvale, CA, USA). Hemoglobin A1c was determined using high-performance liquid chromatography with a VARIANT II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA). Glycosylated albumin was determined using the Glamour 2000 autoanalyzer and an enzyme-based assay (Lucica GA-L; Asahi Kasei Pharma, Tokyo, Japan). Plasma insulin and C-peptide were measured by the chemiluminescent enzyme immunoassay (Insulin Elecsys; Roche Diagnostics, Alameda, CA, USA). The total serum cholesterol (TC), triglyceride (TG), high-density lipoprotein and low-density lipoprotein, alanine transaminase, aspartate aminotransferase, creatinine and uric acid concentrations were determined enzymatically (7600-020; Hitachi, Tokyo, Japan). C-reactive protein (CRP) serum levels were determined using a commercially available latex particle-enhanced immunonephelometry assay (Dade Behring Inc., Newark, NJ, USA). Inter- and intra-assay coefficients of variation were <5% for the measurements. MFG-E8 and TNF- α levels were determined using commercially available enzyme-linked immunosorbent assays, according to the manufacturer's instructions (Cloud-Clone Corp. Houston, TX, USA). Inter- and intra-assay coefficients of variation were <10% and <12%, respectively. All samples were run in duplicate and repeated if there was a > 15% difference between duplicates. The lower and upper limits of detection of the enzyme-linked immunosorbent assay for MFG-E8 were 31.25 and 2,000 pg/mL, respectively, and for TNF- α were 6.25 and 1.00 pg/mL, respectively.

Insulin resistance and insulin secretion evaluation

The homeostasis model assessment (HOMA) was used to estimate steady-state β -cell secretion (HOMA- β) and insulin resistance (HOMA-IR). $\text{HOMA-}\beta = (20 \times \text{Ins}0)/(\text{Glu}0 - 3.5)^{21}$ and $\text{HOMA-IR} = (\text{Ins}0 \times \text{Glu}0)/22.5^{22}$. Ins0 is fasting insulin levels and Glu0 is fasting glucose levels. Insulin sensitivity index (ISI) was calculated from OGTT according to Matsuda and DeFronzo (ISI_M) as follows: $\text{Matsuda (ISI}_M) = 10,000/([\text{Glu}0 \times \text{Ins}0] \times [\text{Glu}_{\text{mean}} \times \text{Ins}_{\text{mean}}])^{1/223}$, which showed better correlation with insulin sensitivity derived using the euglycemic-hyperinsulinemic clamp technique than the HOMA-IR in pregnant women²⁴. The Stumvoll insulin secretion was derived using multiple linear regression models to predict directly measured first- and second-phase insulin release during hyperglycemic clamp studies^{25,26}. The Stumvoll first-phase insulin secretion: (1st-phase secretion) = $(1,194 + [4.724 \times \text{Ins}0] -$

$[117.0 \times \text{Glu}60] + [1.414 \times \text{Ins}60])$ and second-phase insulin response: (2nd-phase secretion) = $(295 + [0.349 \times \text{Ins}60] - [25.72 \times \text{Glu}60] + [1.107 \times \text{Ins}0])$.

Finally, as the limitations of insulin secretion indices (such as HOMA- β and Stumvoll) without controlling for the prevailing insulin resistance, we also calculated the insulin secretion sensitivity index using the standard OGTT, introduced by Renakaranand validated in pregnancy²⁷, which can be considered as a proxy for the disposition index (DI) and as a comprehensive index of β -cell function in pregnancy. DI is defined as $\text{DI}_{1\text{st}} = \text{Stumvoll}_{1\text{st}} \times \text{ISI}_M$; $\text{DI}_{2\text{nd}} = \text{Stumvoll}_{2\text{nd}} \times \text{ISI}_M$.

Statistical analysis

All statistical analyses were carried out using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Data are shown as mean \pm standard deviation or median (25th and 75th percentiles) for continuous variables, or as percentage for categorical variables. When necessary, data that were not normally distributed (as determined using the Kolmogorov-Smirnov test) were log₁₀ or ln transformed to achieve a satisfactory fit to the normal distribution or variance homogeneity. Unpaired independent Student's *t*-test was used to compare differences between two groups, one-way ANOVA or ANCOVA (adjusting for age, pre-BMI, SP, DP, TC and TG) with post-hoc analysis using Bonferroni correction was used to compare significant differences between groups. Categorical variables were examined by the χ^2 -test. Relationships among MFG-E8 and clinical parameters were examined by the calculation of Pearson's correlation and partial correlation coefficients. Bonferroni's correction was used to adjust *P*-values for multiple comparisons. Multivariate regression models were fit for MFG-E8 as a dependent variable, and only variables significantly related ($P < 0.05$) to MFG-E8 by Pearson's correlation analyses were entered into the multiple linear stepwise regression analysis. Participants were divided into tertiles based on MFG-E8 levels, and logistic regression analysis was carried out to create univariate and multivariate models for the odds of developing GDM. A two-sided value of $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics description

A total of 136 pregnant women (66 with GDM and 70 with p-NGT) in gestational weeks 24–28, as well as 44 healthy lean age-matched female controls (CON) were included in the present study. Anthropometric and biochemical characteristics of the subgroups studied are summarized in Table 1.

Women with p-NGT and GDM were well matched with respect to age, gestational days and BMI. Pregnant women showed poor glucose homeostasis and strong insulin resistance, evidenced by significantly increased insulin levels and HOMA-IR ($P < 0.01$ or $P < 0.05$ vs CON), which were further altered in GDM patients ($P < 0.01$ or $P < 0.05$ vs p-NGT). Basic β -cell secretory capacity was reserved, as suggested by increased HOMA- β , $\text{Stumvoll}_{1\text{st}}$ and $\text{Stumvoll}_{2\text{nd}}$ in

Table 1 | Anthropometric parameters and biochemical characteristics of healthy non-pregnant controls, women with normal pregnancy and women with gestational diabetes mellitus

Variables	CON	p-NGT	GDM	P-value		
				CON vs p-NGT	p-NGT vs GDM	CON vs GDM
<i>n</i>	44	70	66			
Age (years)	29.39 ± 4.57	29.10 ± 3.92	29.83 ± 4.66	1.0	1.0	0.986
BMI before pregnancy (kg/m ²)	21.06 ± 2.18	20.45 ± 2.38	21.24 ± 2.70	0.648	1.0	1.0
BMI at blood collection (kg/m ²)		23.59 ± 2.49	24.34 ± 2.77		0.101	
Gestation days at OGTT (days)		194.43 ± 11.05	194.41 ± 11.53		0.992	
Systolic blood pressure (mmHg)	112.27 ± 12.29	111.94 ± 11.08	117.42 ± 12.50	1.0	0.085	0.025
Diastolic blood pressure (mmHg)	72.59 ± 8.55	66.59 ± 8.17	69.08 ± 9.87	0.002	0.135	0.320
TC (mmol/L)	4.10 (3.67–4.32)	4.48 (3.96–5.23)	4.68 (4.23–5.29)	0.013	1.0	0.007
TG (mmol/L)	0.93 (0.78–1.21)	1.32 (1.04–1.73)	1.66 (1.30–2.46)	0.001	0.078	<0.001
HDL (mmol/L)	1.64 ± 0.37	1.61 ± 0.30	1.51 ± 0.35	0.343	0.508	0.032
LDL (mmol/L)	2.56 ± 0.60	2.32 ± 0.88	2.53 ± 0.81	<0.001	0.552	<0.001
ALT (U/L)	12.50 (8.25–15.00)	11.00 (8.00–17.00)	12.00 (8.75–21.00)	1.0	1.0	1.0
AST (U/L)	19.00 (15.00–20.00)	17.00 (14.00–20.00)	17.00 (14.00–20.25)	1.0	1.0	1.0
Cr (umol/L)	50.20 ± 10.48	45.14 ± 7.14	43.20 ± 6.23	0.048	0.813	0.009
HbA1c (%) (mmol/mol)	5.28 ± 0.38 (34.24 ± 4.20)	4.8 ± 0.27 (29.00 ± 2.80)	5.02 ± 0.36 (31.36 ± 4.00)	<0.001	0.007	0.005
GA (%)	13.65 ± 1.94	11.26 ± 1.23	11.46 ± 1.45	<0.001	0.181	<0.001
PG0 (mmol/L)	4.70 ± 0.40	4.50 ± 0.33	5.13 ± 0.65	0.170	<0.001	0.001
PG60 (mmol/L)	7.04 ± 1.69	7.65 ± 1.25	9.92 ± 1.89	0.568	<0.001	<0.001
PG120 (mmol/L)	5.23 ± 1.14	6.14 ± 1.14	8.06 ± 1.94	0.122	<0.001	<0.001
INS0 (μU/mL)	6.26 (5.22–7.91)	8.63 (6.81–11.38)	11.16 (7.28–16.25)	0.001	0.110	<0.001
ISI _M	6.97 ± 2.49	5.83 ± 2.73	4.07 ± 1.97	0.844	0.001	<0.001
HOMA-β	106.65 (81.83–154.35)	178.41 (140.36–233.05)	142.32 (101.09–210.44)	<0.001	0.002	0.105
HOMA-IR	1.28 (1.04–1.73)	1.72 (1.32–2.37)	2.51 (1.68–3.55)	0.017	0.002	<0.001
Stumvoll 1st-phase secretion (pmol/L)	1,239.36 (1,028.85–1,457.89)	1,253.84 (1,059.77–1,539.46)	1,202.09 (955.82–1,520.61)	0.573	1.0	0.616
Stumvoll 2nd-phase secretion (pmol/L)	329.26 (273.20–380.48)	331.00 (285.60–398.84)	329.54 (264.75–397.80)	1.0	0.059	0.153
DI _{1st}	8255.00 (6,460.58–10,973.78)	6640.77 (5,610.11–8,099.58)	4,738.90 (3,597.20–5,822.82)	0.017	<0.001	<0.001
DI _{2nd}	2147.47 (1,712.20–2,821.97)	1,759.84 (1,479.22–2,135.92)	1,246.02 (958.48–1,523.05)	0.018	<0.001	<0.001
CRP (mg/L)	0.24 (0.18–0.30)	1.99 (1.23–3.10)	2.67 (1.77–3.95)	<0.001	<0.001	<0.001
TNF-α (pg/mL)	14.82 ± 3.65	27.31 ± 9.99	35.13 ± 12.63	<0.001	<0.001	<0.001
MFG-E8 (pg/mL)	104.32 (103.94–116.49)	150.36 (139.10–164.03)	183.02 (169.32–235.05)	0.024	0.016	<0.001

Data are mean ± standard deviation, % or median (25th and 75th percentiles). The skewed distributions were log₁₀ or ln transformed for comparison. ANCOVA with post-hoc analysis with Bonferroni's correction was used to compare significant differences between groups. Differences between two groups were tested by unpaired independent Student's *t*-test. ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; CON, healthy non-pregnant controls; Cr, creatinine; CRP, C-reactive protein; DI, disposition index; GA, glycosylated albumin; GDM, gestational diabetes mellitus; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein cholesterol; HOMA-β, homeostasis model assessment of insulin secretion; HOMA-IR, homeostasis model assessment of insulin resistance; INS, insulin levels; ISI_M, Matsuda index; LDL, low-density lipoprotein cholesterol; MFG-E8, milk fat globule-epidermal growth factor 8; OGTT, 75-g oral glucose tolerance test; PG, plasma glucose levels; p-NGT, pregnancy with normal glucose tolerance; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-α.

normal pregnancy, whereas β-cell secretory capacity was shown to decrease as a result of progression to diabetes. Remarkably, after adjusting for HOMA-IR, DI_{1st} and DI_{2nd}

showed a significant reduction in p-NGT compared with CON, which was further decreased in the GDM group (*P* < 0.01).

Table 2 | Pearson's and partial correlations of milk fat globule-epidermal growth factor 8 with anthropometric parameters and biochemical characteristics as well as glucose metabolism in pregnant participants

Variables	Pregnancies		Age, pre-BMI, SP, DP, TC and TG adjusted			
	<i>r</i>	<i>P</i> -value	Bonferroni		<i>r</i>	<i>P</i> -value
			<i>P</i> -value [†]	<i>P</i> -value [†]		
Age	0.208	0.015	1.0			
BMI before pregnancy	0.141	0.102	1.0			
BMI BMI at blood collection	0.201	0.019	0.532	0.145	0.105	1.0
Gestation days at OGTT	-0.153	0.076	1.0	-0.133	0.138	1.0
Systolic blood pressure	0.298	<0.001	<0.001			
Diastolic blood pressure	0.238	0.006	0.168			
TC	0.234	0.007	0.196			
TG	0.216	0.012	0.336			
HDL	0.186	0.032	0.896	0.181	0.043	1.0
LDL	0.231	0.007	0.175	0.003	0.971	1.0
ALT	0.143	0.100	1.0	0.054	0.545	1.0
AST	0.046	0.600	1.0	-0.036	0.691	1.0
Cr	0.102	0.237	1.0	0.083	0.354	1.0
HbA1c	0.394	<0.001	<0.001	0.324	<0.001	<0.001
GA	-0.002	0.978	1.0	0.033	0.714	1.0
PG0	0.453	<0.001	<0.001	0.358	<0.001	<0.001
PG60	0.383	<0.001	<0.001	0.294	0.001	0.028
PG120	0.291	0.001	0.028	0.204	0.022	0.616
INS0	0.348	<0.001	<0.001	0.253	0.004	0.112
ISI _M	-0.376	<0.001	<0.001	-0.322	<0.001	<0.001
HOMA-β	-0.065	0.450	1.0	-0.071	0.429	1.0
HOMA-IR	0.405	<0.001	<0.001	0.309	<0.001	<0.001
Stumvoll 1st-phase secretion	0.092	0.286	1.0	0.063	0.485	1.0
Stumvoll 2nd-phase secretion	-0.084	0.329	1.0	-0.111	0.217	1.0
DI _{1st}	-0.451	<0.001	<0.001	-0.320	<0.001	<0.001
DI _{2nd}	-0.447	<0.001	<0.001	-0.331	0.000	<0.001
CRP	-0.137	0.112	1.0	-0.169	0.058	1.0
TNF-α	-0.280	0.001	0.028	-0.346	<0.001	<0.001

Skewed distributions were log₁₀ or ln transformed. Correlation between variables was analyzed by Pearson's correlation test and partial correlation test (adjusted by age, sex, body mass index [BMI], systolic blood pressure [SP], diastolic blood pressure [DP], total cholesterol [TC] and triglyceride [TG]). Bonferroni's correction was applied for multiple testing (28 times) to adjust *P*-value[†]. ALT, alanine transaminase; AST, aspartate aminotransferase; CON, healthy non-pregnant controls; Cr, creatinine; CRP, C-reactive protein; DI, disposition index; GA, glycosylated albumin; GDM, gestational diabetes mellitus; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein cholesterol; HOMA-β, homeostasis model assessment of insulin secretion; HOMA-IR, homeostasis model assessment of insulin resistance; INS, insulin levels; ISI_M, Matsuda index; LDL, low-density lipoprotein cholesterol; MFG-E8, milk fat globule-epidermal growth factor 8; OGTT, 75-g oral glucose tolerance test; PG, plasma glucose levels; pre-BMI, body mass index before pregnancy; p-NGT, pregnancy with normal glucose tolerance; TNF-α, tumor necrosis factor-α.

The median (25th and 75th percentiles) plasma MFG-E8 level in all participants was 153.79 pg/mL (123.56–189.25 pg/mL). Circulating plasma concentrations of MFG-E8 were significantly higher in pregnant participants, and were further increased in GDM patients, after correcting for age, pre-BMI, SP, DP, TC and TG, as shown in Table 1 (all *P* < 0.05).

Correlation of MFG-E8 with clinical parameters analysis

Next, the relationship of circulating MFG-E8 levels with various anthropometric parameters was investigated using Pearson's and partial correlations. MFG-E8 levels were neither related to

pre-BMI, alanine transaminase, aspartate aminotransferase and glycosylated albumin nor to insulin secretion indices evaluated by HOMA-β, Stumvoll_{1st}, and Stumvoll_{2nd}, as summarized in Table 2, and Tables S1 and S2.

No correlation was found between MFG-E8 plasma levels and any variable in the control group (Table S1). In all pregnant participants, MFG-E8 had a significantly positive correlation with age, BMI at blood collection, blood pressures and lipid profiles (Figure 1). Nevertheless, the correlation was no longer significant after Bonferroni's correction was carried out, except for SP. In addition, MFG-E8 correlated positively with hemoglobin A1c,

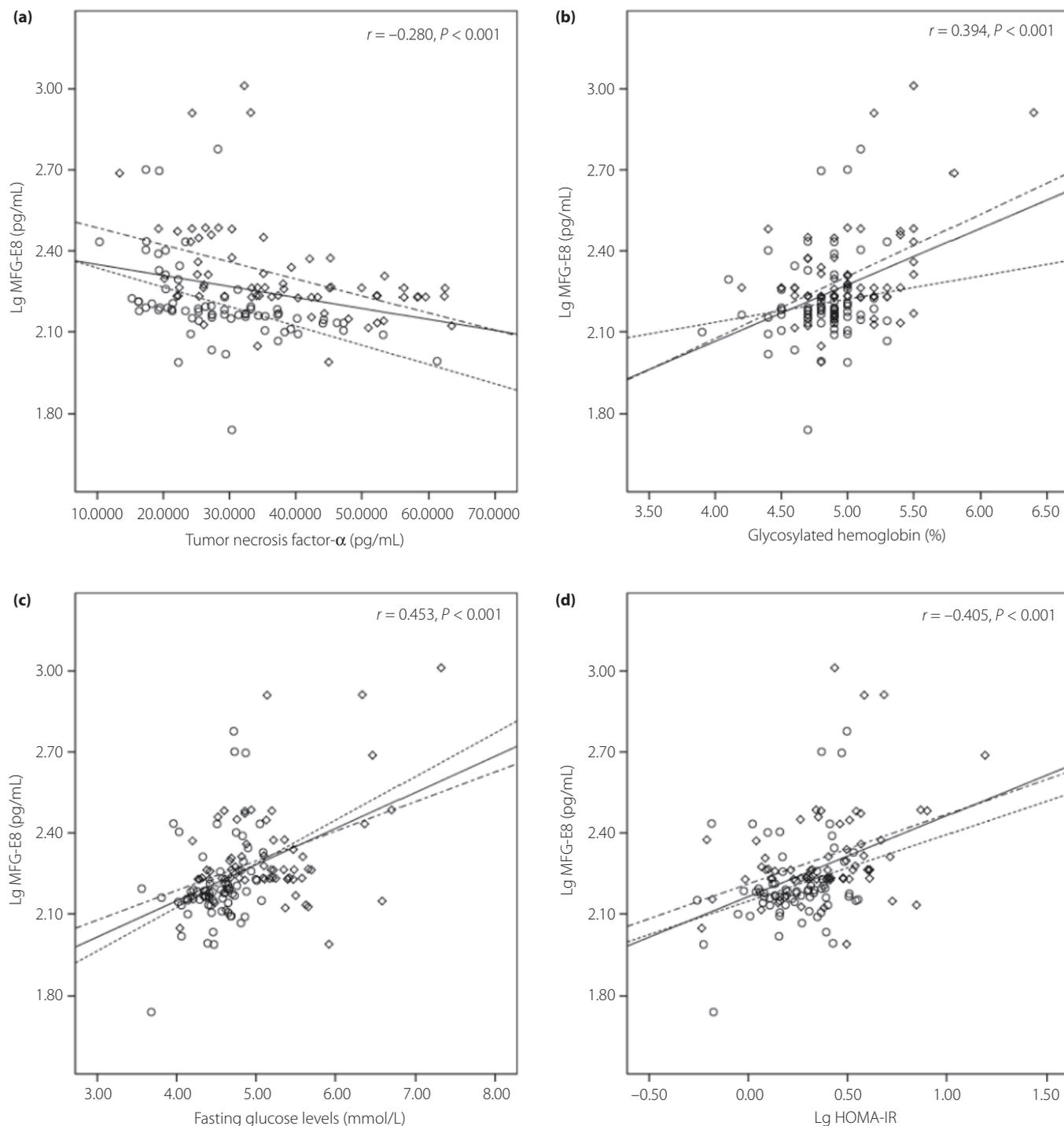


Figure 1 | Correlation of plasma milk fat globule-epidermal growth factor 8 (MFG-E8) levels (log₁₀ transformed) with (a) tumor necrosis factor- α levels, (b) glycosylated hemoglobin, (c) fasting glucose levels (d) homeostasis model assessment of insulin resistance, log₁₀ transformed (Lg HOMA-IR), (e) insulin sensitivity index, (f) disposition index of first phase insulin secretion, log₁₀ transformed (Lg DI_{1st}) and (g) disposition index of second phase insulin secretion, log₁₀ transformed (Lg DI_{2nd}). Circles represent normal pregnancy, diamonds represent gestational diabetes mellitus.

fasting and postprandial glucose levels, fasting insulin levels, and HOMA-IR, whereas it was inversely correlated with ISI_M and insulin secretion evaluated by disposition indices (DI_{1st} and

DI_{2nd}). It is noteworthy that all of these correlations remained statistically significant, despite being adjusted for age, pre-BMI, SP, DP, TC and TG. After Bonferroni's correction was applied,

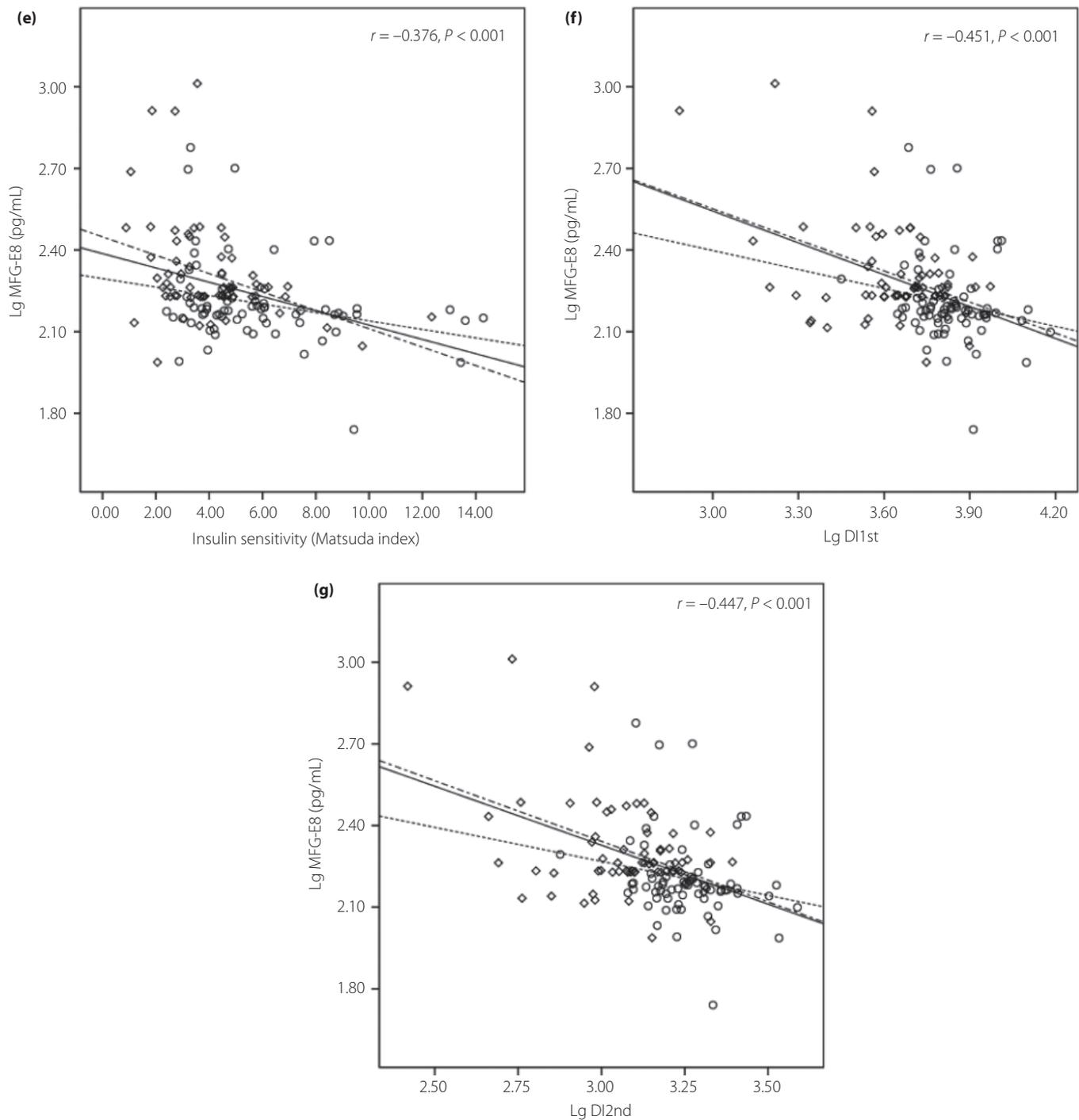


Figure 1 | Continued.

the statistically significant association persisted, except for glucose levels at 120 min and fasting insulin levels.

However, the situation was different after subgroup analysis. MFG-E8 levels were positively associated with age, BMI at blood collection, lipid profiles, fasting glucose and insulin levels, and HOMA-IR in the normal pregnant group, whereas MFG-E8 levels were inversely associated with ISI_M and disposition

indices in the same group. However, all parameters except BMI at blood collection lost the statistically significant correlation with MFG-E8 after adjusted for age, pre-BMI, SP, DP, TC and TG. In the diabetic group, a positive correlation between MFG-E8 and blood pressure, hemoglobin A1c, fasting and postprandial glucose levels, fasting insulin levels, HOMA-IR, and creatinine, as well as a negative association with gestation days,

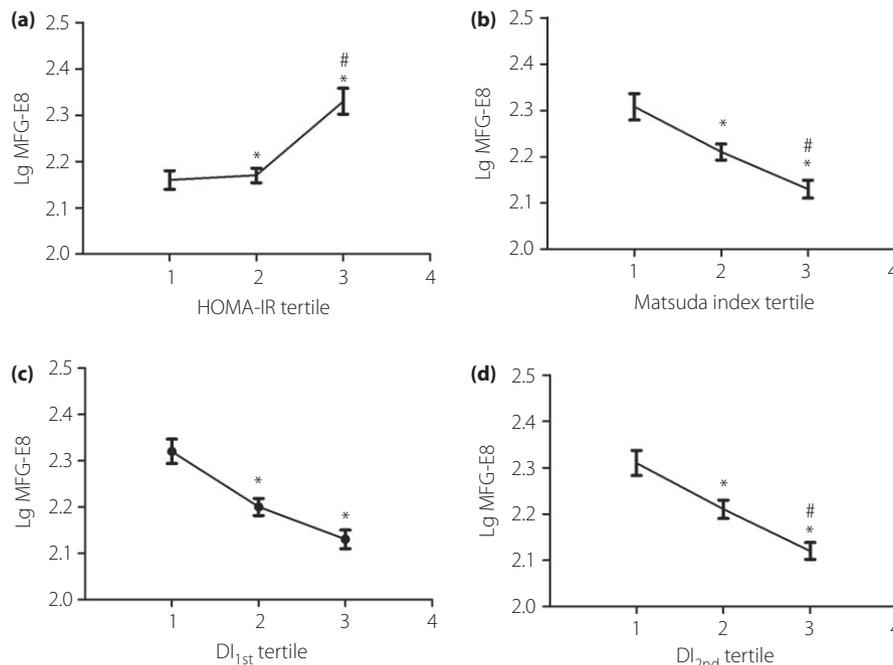


Figure 2 | Log₁₀ transformed milk fat globule-epidermal growth factor 8 (LgMFG-E8) in tertiles of (a) homeostasis model assessment of insulin resistance (HOMA-IR), (b) Matsuda index, (c) disposition index of first phase insulin secretion (DI_{1st}) and (d) disposition index of second phase insulin secretion (DI_{2nd}). Data were shown as mean ± standard error of the mean. MFG-E8 was logarithmically transformed for comparison. Differences between groups were assessed by one-way ANCOVA adjusted for age, pre-pregnancy body mass index, systolic blood pressure, diastolic blood pressure, total serum cholesterol and triglyceride. **P* < 0.05 vs the first tertile, #*P* < 0.05 vs the second tertile.

ISI_M and disposition indices were observed, and the association remained statistically significant after interference factors were adjusted, except for glucose levels at 120 min, fasting insulin levels and HOMA-IR. After Bonferroni's correction was applied for multiple testing (28 times) to adjust *P*-values, all significance was eliminated.

There was a significantly negative correlation between circulating MFG-E8 and TNF- α in all pregnancies or subgroups. After Bonferroni correction was carried out and interference factors were adjusted, the statistical significance still remained. In addition, MFG-E8 was negatively correlated with CRP only in the GDM group, and lost statistical significance after multiple comparison.

When participants were divided by tertiles of HOMA-IR, ISI_M and disposition indices, MFG-E8 concentration increased progressively from the first to the third tertile of HOMA-IR, whereas MFG-E8 concentration decreased progressively from the first to the third tertile of ISI_M, DI_{1st} and DI_{2nd} (all *P* < 0.05; Figure 2).

Multiple regression analysis

The multivariate stepwise linear regression analysis showed that independent factors influencing MFG-E8 levels were fasting glucose levels ($\beta = 0.092$; *P* < 0.001), DI_{1st} ($\beta = -0.300$; *P* < 0.001) and TNF- α ($\beta = -0.005$; *P* < 0.001). The adjusted *R*² value was 0.371.

Logistic regression analysis

Finally, pregnant women were stratified into tertiles based on MFG-E8 levels; women in the third tertile had an 8.62-fold risk of developing GDM compared with women in the first tertile. This odds ratio merely decreased a 0.37-fold risk after adjustment for age, pre-BMI, SP, DP, TC and TG, and remained statistically significant (*P* < 0.01; Figure 3).

DISCUSSION

To the best of our knowledge, this is the first study to show that plasma MFG-E8 levels were significantly increased in pregnant women vs non-pregnant women (*P* < 0.05). This suggests a similarity in alteration in MFG-E8 activity that was found in the myometrium, as well as in the endometrium during human pregnancy^{17,28}, which was probably due to MFG-E8 functioning as an extracellular growth factor during gestation that promoted expansion of the connective tissue framework supporting the hypertrophied bundles of smooth muscle. Furthermore, we established that the plasma concentrations of MFG-E8 were greater in GDM than that observed in normal pregnant women, which was in consist with the elevated MFG-E8 levels that have been confirmed in *db/db* mice and type 2 diabetes patients^{29,30}. However, the exact mechanism of elevated MFG-E8 concentrations during human gestation and GDM has not been completely elucidated yet, and further research is required for it to be clearly understood. In the current study, when

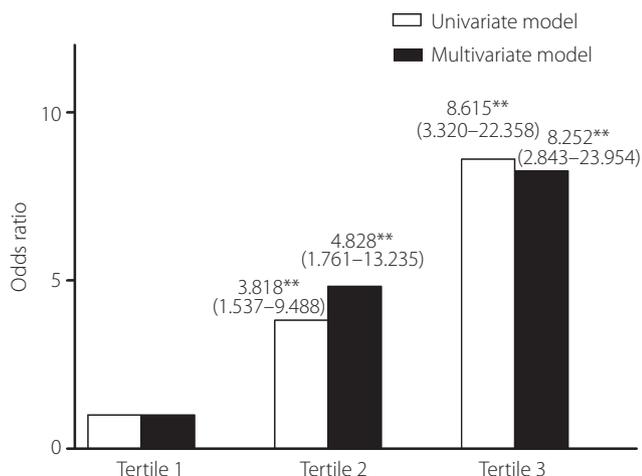


Figure 3 | Odds of developing gestational diabetes mellitus by milk fat globule-epidermal growth factor 8 (MFG-E8) tertiles. Participants were divided into tertiles based on the MFG-E8 levels. Univariate (white) and multivariate (dark) logistic regression analyses were used to determine odds ratios for the development of gestational diabetes mellitus in each tertile. The multivariate logistic regression model includes adjustment for age, pre-pregnancy body mass index, systolic blood pressure, diastolic blood pressure, total serum cholesterol and triglyceride. **Significantly different from the reference tertile at the $P < 0.01$ level.

pregnancies were divided into tertiles based on MFG-E8 levels, women with the highest levels of MFG-E8 showed a markedly elevated risk of GDM compared with women with the lowest MFG-E8 levels. This estimate of risk was consistent with, but did not prove the involvement of, MFG-E8 in GDM pathophysiology.

In the hypothesized direction, pregnant women were under a state of subclinical chronic inflammation as evidenced by significantly higher TNF- α and CRP levels compared with controls, which were further altered in GDM. MFG-E8 is now accepted as a major inflammatory mediator stimulating anti-inflammatory reprogramming of human and murine endothelial cells and macrophages³¹. MFG-E8 alleviates the inflammation by suppressing lipopolysaccharide-toll like receptor 4 signaling and quenching TNF- α production^{32,33}. MFG-E8 has been reported to alleviate the inflammation, and negatively regulate the production of TNF- α in patients with rheumatoid arthritis, sepsis and ischemia reperfusion injury^{10,34,35}. Consistent with these results, the present study showed that TNF- α was negative, and independently correlated with plasma MFG-E8 concentrations during gestation. However, both MFG-E8 and TNF- α circulating levels were increased during normal gestation and GDM in the present study. The reason for the discrepancy is unclear and is probably because of the complexity in patients during pregnancy, as it has been shown that endometrial MFG-E8 gene expression is significantly upregulated by TNF- α during gestation, which is a critical step required for the endometrial changes during embryonic attachment and invasion³⁶. Future

studies are needed to elucidate this point. Furthermore, contrary to the results of Dai *et al.*⁷ that found circulating MFG-E8 concentrations were negatively associated with CRP in patients with coronary artery disease, we did not find a significant association between MFG-E8 and CRP levels, and were unable to support that conclusion in this current study, which focused on patients with pregnancy and diabetes.

In the current study, normal pregnancy was associated with impaired β -cell function evidenced by enhanced insulin resistance and inadequate insulin secretion. This situation was even worse in women with GDM. Interestingly, as there are few studies available on the topic in general, the present results not only replicated the finding that MFG-E8 was negatively associated with TNF- α , but also showed that MFG-E8 correlated positively with indices of insulin resistance, including HOMA-IR and ISI_M. Importantly, we excluded potentially confusing effects in the association of MFG-E8 with pregnancy and GDM, such as age, BMI, blood pressure and lipid profiles. In addition, we also ruled out the interaction effect for multiple comparisons by carrying out Bonferroni's correction. Nevertheless, the negative correlation of MFG-E8 and insulin secretion was only observed by using disposition indices that reflected accurate insulin secretion controlling for insulin resistance, while the first phase insulin secretion (DI_{1st}) was predictive of MFG-E8 levels during gestation. Furthermore, MFG-E8 concentration increased progressively from the first to the third tertile of HOMA-IR, whereas MFG-E8 concentration increased inversely in ISI_M, DI_{1st} and DI_{2nd}. However, riding on these mechanistic tools, it is not clear whether elevated MFG-E8 level is a compensatory response or a marker of insulin resistance and insulin secretion. Accumulating data suggested that low-grade inflammation was a central feature of the insulin resistance syndrome, and that TNF- α was the most powerful predictor of insulin resistance and was inversely correlated to insulin secretion during pregnancy³⁷. Consistent with plasma MFG-E8 concentrations being negatively correlated with TNF- α in the present study, therefore, we speculated that MFG-E8 might be associated with insulin resistance and insulin secretion through an inflammatory pathway.

Overall, considering the complexity of the mechanisms of MFG-E8 functioning, we cannot exclude the possibility that the MFG-E8 levels might be associated with other unknown intermediate factors, which could affect insulin resistance and insulin secretion. Further studies are required to delineate the mechanisms of MFG-E8 in GDM and insulin resistance. Correlations between MFG-E8 and parameters of β -cell function were not statistically significant in each group individually, which might be due to the relatively small sample size.

There were still some limitations in the study that need to be emphasized. First, our sample size was relatively small. Second, the cross-sectional design of the study prevented us from extracting safe conclusions on the possible etiological relationship between MFG-E8 and pregnancy and diabetes. Third, the study was carried out during the 24–28 weeks of pregnancy without considering the whole duration of gestation. Fourth,

the study excluded the effects of hormone levels, such as prolactin, estrogen, cortisol and progesterone. Larger prospective studies are necessary for the validation of these conclusions and to elucidate the clinical roles of MFG-E8.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Pearson's and partial correlations of milk fat globule-epidermal growth factor 8 with anthropometric parameters and biochemical characteristics as well as glucose metabolism in healthy controls

Table S2 | Pearson's and partial correlations of Milk fat globule-epidermal growth factor 8 with anthropometric parameters and biochemical characteristics as well as glucose metabolism in subgroups of pregnancies