Erythrocyte n-6 Polyunsaturated Fatty Acids, Gut Microbiota, and Incident Type 2 Diabetes: A Prospective Cohort Study

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Diabetes Care 2020;43:2435–2443 | https://doi.org/10.2337/dc20-0631

OBJECTIVE

To examine the association of erythrocyte n-6 polyunsaturated fatty acid (PUFA) biomarkers with incident type 2 diabetes and explore the potential role of gut microbiota in the association.

RESEARCH DESIGN AND METHODS

We evaluated 2,731 participants without type 2 diabetes recruited between 2008 and 2013 in the Guangzhou Nutrition and Health Study (Guangzhou, China). Case subjects with type 2 diabetes were identified with clinical and biochemical information collected at follow-up visits. Using stool samples collected during the follow-up in the subset (n = 1,591), 16S rRNA profiling was conducted. Using multivariable-adjusted Poisson or linear regression, we examined associations of erythrocyte n-6 PUFA biomarkers with incident type 2 diabetes and diversity and composition of gut microbiota.

RESULTS

Over 6.2 years of follow-up, 276 case subjects with type 2 diabetes were identified (risk 0.10). Higher levels of erythrocyte γ-linolenic acid (GLA), but not linoleic or arachidonic acid, were associated with higher type 2 diabetes incidence. Comparing the top to the bottom quartile groups of GLA levels, relative risk was 1.72 (95% CI 1.21, 2.44) adjusted for potential confounders. Baseline GLA was inversely associated with gut microbial richness and diversity (α-diversity, both $P < 0.05$) during follow-up and significantly associated with microbiota β-diversity ($P = 0.002$). α-Diversity acted as a potential mediator in the association between GLA and type 2 diabetes ($P < 0.05$). Seven genera (*Butyrivibrio*, *Blautia*, *Oscillospira*, *Odoribacter*, *S24-7 other*, *Rikenellaceae other*, and *Clostridiales other*) were enriched in quartile 1 of GLA and in participants without type 2 diabetes.

CONCLUSIONS

Relative concentrations of erythrocyte GLA were positively associated with incident type 2 diabetes in a Chinese population and also with gut microbial profiles. These results highlight that gut microbiota may play an important role linking n-6 PUFA metabolism and type 2 diabetes etiology.

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Received 25 March 2020 and accepted 5 July 2020

This article contains supplementary material online at https://doi.org/10.2337/figshare.12624821.

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Type 2 diabetes is one of the most prevalent metabolic conditions worldwide, and 463 million adults are living with diabetes (1). To examine lifestyle-related risk factors for the development of type 2 diabetes, the role of different fatty acids has been an important research agenda. For example, the associations of blood biomarkers of different saturated fatty acids and n-3 polyunsaturated fatty acids (PUFAs) with type 2 diabetes have been investigated in different cohorts including a Chinese cohort used in the current study (2–5). During the past two decades, consumption of n-6 PUFAs, rich in vegetable oil, has been increasing rapidly (6), while their relationship with human health remains controversial. The metabolic pathway of n-6 PUFAs is hypothesized to be closely involved in the type 2 diabetes etiology (7), but has not been well characterized yet.

A recent pooled analysis of 20 prospective cohort studies investigated the association of linoleic acid (LA; 18:2n6) and arachidonic acid (AA; 20:4n6) biomarkers with incident type 2 diabetes, reporting that LA, but not AA, was inversely associated with type 2 diabetes (8). However, in this pooling project, only two individual n-6 PUFA biomarkers were evaluated, while cohorts in Europe reported γ-linolenic acid (GLA) and other n-6 PUFAs to be associated with higher type 2 diabetes risk (8). Moreover, with only one small cohort from Taiwan in the above pooling project (8), evidence from Asian populations has been limited. As an Asian population has different metabolic and lifestyle characteristics in relation to type 2 diabetes compared with American or European populations (9), more investigation for n-6 PUFA biomarkers in Asian populations is highly warranted.

The protective effect of n-6 PUFA or LA, the most abundant n-6 PUFA, on insulin homeostasis has been well characterized and discussed to link n-6 PUFA exposure to lower type 2 diabetes risk (8). However, gut microbiota has not been heavily investigated in research on PUFAs and any noncommunicable diseases. On one hand, gut microbiota has been considered as an important risk factor for type 2 diabetes and insulin resistance (10). On the other hand, rodent studies have suggested that high tissue levels of n-6 PUFAs are associated with differences in gut microbiota, including increased *Proteobacteria*, reduced *Actinobacteria* and *Bacteroidetes*, and altered microbial α-diversity (11,12). Yet, to our knowledge, there are no human cohort studies investigating the potential link among n-6 PUFA exposure, type 2 diabetes risk, and gut microbiota composition and diversity.

Therefore, we hypothesized that long-term exposure to different n-6 PUFA status might be associated with type 2 diabetes risk in the Chinese population, and gut microbiota might partly mediate the above association. Evaluating the prospective cohort in China, we aimed to examine the prospective associations *1*) between n-6 PUFA biomarkers and type 2 diabetes risk in a Chinese population, and *2*) between n-6 PUFA biomarkers and gut microbiota composition and diversity and subsequent health outcomes. As a secondary aim, we also examined the prospective association of dietary n-6 PUFAs with gut microbiota and type 2 diabetes risk in the Chinese population.

RESEARCH DESIGN AND METHODS

Study Design and Population

This study was based on the Guangzhou Nutrition and Health Study, a community-based prospective cohort in southern China. A total of 4,048 Chinese participants aged 40–75 years who lived in Guangzhou for at least 5 years were recruited between 2008 and 2010 (n = 3,169) and 2012 and 2013 (n = 879). All participants have been followed up every 3 years approximately. The study was registered at ClinicalTrials.gov (NCT03179657).

According to our prespecified inclusion and exclusion criteria of our participants for the present analysis (Supplementary Fig. 1), we excluded participants with missing information on age or sex (n = 9), diet (n = 7), self-reported/diagnosed type 2 diabetes (n = 400) or history of cancer (n = 16) at baseline, or extreme levels of self-reported total energy intake (<800 or >4,000 kcal/day for men and <500 or >3,500 kcal/day for women) (n = 53). We also excluded those without measurement of erythrocyte membrane fatty acid compositions at baseline (n = 298) and further excluded those without any follow-up information on type 2 diabetes status (n = 534; follow-up rate 84%). The current analyses were censored by date of type 2 diabetes ascertainment or 30 April 2019, whichever happened first. A total of 2,731 individuals (Han clan) were included in the current study, with a median follow-up time of 6.2 years.

Among the above included 2,731 individuals, stool samples from 1,606 individuals were collected between 2014 and 2018 for the subsequent 16S profiling, with 1,591 individuals included in the microbial association analysis after excluding participants who used antibiotics within 2 weeks before stool collection (n = 15).

Measurement of Erythrocyte Membrane Fatty Acids and Covariates

We considered relative concentrations (percentage of total fatty acids) of erythrocyte LA, GLA, and AA as the main exposure variables. Blood samples were drawn after overnight fasting at the baseline visit. Fatty acid moieties of erythrocyte membranes were *trans*-methylated and measured as proportions (percentage) of total fatty acids by using gas chromatography. Sociodemographic, lifestyle, and clinical covariates were collected with standardized questionnaires, and dietary information was collected using a validated food frequency questionnaire (13). Details of fatty acid and covariate measurements are available in the Supplementary Materials.

Ascertainment of Case Subject With Type 2 Diabetes

At baseline (for exclusion) and follow-up visits, case subjects with type 2 diabetes were ascertained according to the criteria of the American Diabetes Association for type 2 diabetes diagnosis (14) if a participant met one of the following criteria: a fasting blood glucose concentration ≥7.0 mmol/L (126 mg/dL), HbA_{1c} concentration ≥6.5%, or self-reported medical treatment for diabetes.

Fecal Sample Collection and 16S rRNA Profiling

Fecal samples were collected on site during follow-up visits between 2014 and 2018. Microbial DNA extraction, PCR, and amplicon sequencing were performed as described (see Supplementary Materials). FASTQ files were demultiplexed, merge-paired, and quality filtered by Quantitative Insights into Microbial Ecology software (version 1.9.0) (15). Sequences were clustered into operational taxonomic units (OTUs) with 97% similarity and annotated based on the Greengenes Database (version 13.8) (16).

Statistical Analysis

Statistical analyses were performed using Stata 15 (StataCorp, College Station, TX) or R (version 3.6.3). We used multivariable Poisson regression models to estimate the risk ratio (RR) and 95% CIs of type 2 diabetes comparing quartiles of the erythrocyte n-6 PUFA (LA, GLA, and AA), adjusting for potential confounders. We fitted three statistical models: model 1 included age, sex, BMI, and waist-to-hip ratio (WHR); model 2 was model 1 plus education, household income, smoking, alcohol drinking status, physical activity, total energy intake, and family history of diabetes; and model 3 was model 2 plus baseline erythrocyte total n-3 PUFAs and fasting glucose. *P* value for trend was calculated based on per-quartile difference in the corresponding n-6 PUFA variable. To compare our findings above with traditional risk factors of type 2 diabetes, we took BMI as an example and performed a post hoc analysis to calculate RRs of type 2 diabetes comparing quartiles of BMI using Poisson regression, adjusted for the same covariates in model 3 described above.

In sensitivity analyses, we repeated the above analysis based on model 3 to examine the impact of loss to follow-up by a simple imputation (assuming that participants lost to follow-up did not develop type 2 diabetes) and 10 rounds of multiple imputations based on regression model using all covariates listed in model 3. In addition, we excluded case subjects with type 2 diabetes ascertained only by fasting glucose to test the potential influence of misclassification of type 2 diabetes by fasting glucose.

We assessed influences of additional potential covariates on the models: dietary variables (dietary intake of dairy products, red and processed meat, fish, vegetables, and fruit, in quartiles), blood lipids (triglycerides and LDL), prevalent coronary heart disease, and treatment for hypertension or hyperlipidemia. We further examined the potential interaction of erythrocyte n-6 PUFA biomarkers with age, sex, BMI, or n-3 PUFA biomarker concentrations on type 2 diabetes risk. Post hoc–stratified analyses were performed if there was a significant interaction ($P_{interaction} < 0.05$) for age, sex, BMI, or n-3 PUFA biomarker concentrations with the n-6 PUFA biomarkers.

To test our hypothesis that gut microbiota may partly mediate the association between n-6 PUFAs and type 2 diabetes, we calculated the α-diversity metrics (observed OTUs, Shannon diversity index, Chao index, and Simpson index). Observed OTUs and Chao index represent the microbial richness (the number of different features in a sample), and Shannon diversity index and Simpson index represent diversity (how evenly the microbes are distributed in a sample). We standardized α-diversity indicators (*z* score was calculated: the variable was subtracted by the mean and divided by the SD) and then reduced assay-dependent variabilities by fitting a linear mixed model with an α-diversity indicator as an outcome variable and with assay-specific covariates including sequencing depth and Bristol scale as fixed effects and sequencing batch as a random effect. The residuals from this modeling were then used in the following n-6 PUFA biomarker association analyses. We used a linear regression model to examine the association of individual n-6 PUFA biomarkers (by quartile) with the four α-diversity metrics, adjusted for covariates in model 3. We also conducted an additional adjustment for dietary fiber intake to assess the potential marginal effect of dietary fiber, as fiber is a well-known factor influencing the gut microbiome (17). To investigate the relevance of significant findings in relation to type 2 diabetes, we performed mediation analysis (R {*mediation*}) (18) with the residuals of α-diversity as a mediator of the relationship between n-6 PUFAs and type 2 diabetes, adjusting for all covariates described in model 3. We examined the cross-sectional associations between the residuals of α-diversity metrics and type 2 diabetes by using logistic regression models, adjusted for age, sex, BMI, education, household income, smoking status, alcohol drinking status, and prevalent hypertension and dyslipidemia.

We conducted a principal coordinate analysis and permutational multivariate ANOVA (PERMANOVA) (R function adonis {*vegan*}, 999 permutations) (19) based on Bray Curtis distance to compare the whole gut composition (β-diversity, how different are the microbial communities in one environment compared with another) at genus level by quartiles of n-6 PUFA biomarkers. The potential confounders included in the PERMANOVA were sequencing depth, sequencing batch, and Bristol scale, plus covariates in model 3. The above analysis was repeated for gut composition at OTU level. Scaled relative abundances (i.e., divided by the SD) were used to lessen the influence of highly abundant genera on Bray Curtis distance. The association of community structure with type 2 diabetes was also assessed by PERMANOVA (R function adonis, 999 permutations) to obtain R^2, indicating the proportion of the variability explained by the studied variables, including n-6 PUFAs. We performed the post hoc pairwise comparisons between different quartiles of GLA with Bonferroni correction (cutoff $P < 0.008$).

At genus level, we performed a linear discrimination analysis (LDA) as implemented using LEfSe (20). The default parameters were used (α value for Wilcoxon tests was 0.05, and the LDA score was 2.0) to identify biomarkers at genus level, distinguishing different quartiles of n-6 PUFAs or different type 2 diabetes status. Only taxon present in at least 10% of samples were included in the analyses. Besides, we evaluated the potential correlation of the above identified genera with type 2 diabetes–related traits, including fasting insulin, glucose, total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, non-HDL cholesterol, HbA_{1c}, HOMA of insulin resistance, and HOMA of β-cell function. We first modeled type 2 diabetes–related traits as outcome variables in linear regression with age, sex, and BMI and obtained the residuals of each type 2 diabetes–related trait, respectively, and then we calculated the Spearman correlation coefficients between the genera and the residuals of type 2 diabetes–related traits. Results displaying a *P* value <0.05 after Bonferroni adjustment were considered significant.

As a secondary analysis, we examined the associations between dietary n-6 PUFAs (LA and AA) and type 2 diabetes using model 3. Then, the same model was used to examine the associations between dietary n-6 PUFAs and gut microbial diversity as the above analyses of erythrocyte n-6 PUFAs. Dietary GLA was not included in the analysis, as the level of GLA in the diet is very low and less common in Chinese foods. The Spearman correlation coefficients of the dietary n-6 PUFAs with erythrocyte n-6 PUFAs were calculated.

RESULTS

Characteristics of the Study Participants

Table 1 and Supplementary Table 1 summarize the baseline characteristics of the population by quartiles of erythrocyte

Table 1—Baseline population characteristics by quartiles of erythrocyte GLA (n = 2,731)

	GLA (γC18:3n6)			
	Q1 (N = 682)	Q2 (N = 683)	Q3 (N = 683)	Q4 (N = 683)
Age (years)	57.9 (6.0)	58.1 (5.8)	58.3 (5.6)	58.1 (5.2)
Sex (% of women)	227 (33)	231 (34)	201 (29)	167 (24)
BMI (kg/m^2)	22.7 (3.1)	23.2 (2.9)	23.5 (3.0)	23.5 (3.1)
WHR	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)
Education level				
Middle school or lower	176 (26)	182 (27)	198 (29)	209 (31)
High school or professional college	338 (50)	327 (48)	317 (46)	303 (44)
University	168 (25)	174 (25)	168 (25)	171 (25)
Household income (Chinese Yuan/month/person)				
≤500	10 (1)	16 (2)	13 (2)	16 (2)
500–1,500	159 (23)	162 (24)	195 (29)	188 (28)
1,500–3,000	429 (63)	407 (60)	362 (53)	358 (52)
>3,000	84 (12)	98 (14)	113 (17)	121 (18)
Family history of diabetes	71 (10)	62 (9)	76 (11)	78 (11)
Current smoking	110 (16)	108 (16)	104 (15)	90 (13)
Current alcohol drinking	48 (7)	59 (9)	32 (5%)	35 (5)
Physical activity (MET · h/day)	40.5 (14.0)	42.3 (15.1)	41.4 (15.3)	41.7 (15.1)
Total energy intake (kcal/day)	1,759 (491)	1,778 (484)	1,771 (499)	1,764 (471)
Dairy intake (g/day)	16.1 (13.2)	17.3 (14.5)	16.7 (15.9)	16.1 (13.6)
Red and processed meat intake (g/day)	84.5 (54.4)	84.7 (52.9)	82.4 (53.0)	84.0 (53.4)
Vegetable intake (g/day)	374.0 (189.9)	388.3 (188.0)	383.9 (260.8)	383.2 (248.2)
Fruit intake (g/day)	149.8 (107.1)	149.2 (116.6)	147.0 (112.1)	145.3 (104.6)
Dietary fiber intake (g/day)	11.2 (3.2)	11.3 (3.1)	11.4 (4.6)	11.3 (4.4)
Fish intake (g/day)	56.7 (69.0)	50.6 (38.5)	49.1 (34.16)	49.0 (63.7)
n-3 PUFAs (%)	7.7 (1.6)	7.1 (1.6)	6.8 (1.7)	6.0 (2.0)
Fasting blood glucose (mmol/L)	4.7 (0.6)	4.7 (0.7)	4.7 (0.7)	4.7 (0.7)
Serum TG (mmol/L)	1.2 (0.9)	1.4 (0.8)	1.7 (1.1)	1.8 (1.4)
Serum HDL (mmol/L)	1.5 (0.3)	1.4 (0.3)	1.4 (0.3)	1.3 (0.3)
Serum LDL (mmol/L)	3.5 (0.8)	3.5 (0.8)	3.6 (0.9)	3.6 (1.0)

Data are mean (SD) for continuous measures and n (%) for categorical measures. TG, triglycerides.

GLA, LA, and AA. The median levels (interquartile range) of baseline erythrocyte LA, GLA, AA, and total n-6 PUFAs (percent total fatty acids) were 9.84 (8.89–10.76), 0.037 (0.025–0.052), 11.51 (10.00–12.60), and 21.6 (19.6–23.1), respectively. Supplementary Tables 2 and 3 present baseline population characteristics of participants with and without follow-up information and 16S profiling, respectively. Participants who dropped out had higher serum glucose levels, consumed fewer vegetables and energy, tended to be older, less educated, and less physically active, and were more likely to smoke. Dietary LA intake was positively (r = 0.11; $P < 0.001$) correlated with erythrocyte LA (Supplementary Table 4). Different food sources of n-6 PUFAs were summarized in the Supplementary Table 5.

Association of Erythrocyte n-6 PUFAs With Incident Type 2 Diabetes

Higher levels of baseline GLA were associated with higher risk of type 2 diabetes across the three statistical models (P trend <0.001 for all models) (Table 2). For model 3, compared with the first quartile group (Q1), RRs (95% CIs) of type 2 diabetes at Q2, Q3, and Q4 were 1.22 (0.85, 1.74), 1.43 (1.01, 2.03), and 1.72 (1.21, 2.44), respectively. For BMI, the multivariable-adjusted RR of type 2 diabetes (95% CI) of Q4 was 2.59 (1.74, 3.86) compared with Q1 (Supplementary Fig. 2). No association was found for LA or AA. Similar results (Supplementary Tables 6 and 7) were obtained after using the simple or multiple imputation for the missing data or excluding cases ascertained only by fasting glucose (n = 52) (Supplementary Table 8) in the sensitivity analysis. Adjustment for additional dietary factors and disease histories showed similar results (Supplementary Table 9). We did not observe significant interaction for n-6 PUFA biomarkers with age, sex, BMI, or n-3 PUFAs on type 2 diabetes risk ($P_{interaction} > 0.05$).

Prospective Association of Erythrocyte n-6 PUFA Biomarkers With Gut Microbiota Diversity

Lower GLA was associated with higher gut microbiota α-diversity (Q4 vs. Q1, P = 0.021 for observed OTUs, 0.028 for Shannon diversity index, and 0.023 for Chao index, respectively) (Fig. 1*A*). However, baseline LA, AA, or total n-6 PUFAs were not associated with the microbial α-diversity (Supplementary Table 10). These results remained similar after further adjusting for dietary fiber intake (Supplementary Table 11). Among 1,591 individuals included in the microbial association analysis, we also observed positive association between GLA and type 2 diabetes (Supplementary Table 12). α-Diversity acted as a potential partial mediator of the association between GLA and type 2 diabetes (7.9%, 7.1%, and 7.1% of mediation for observed OTUs, Shannon diversity index, and Chao index, respectively, $P < 0.05$) (Supplementary Table 13).

Table 2—Association of erythrocyte n-6 fatty acids with incident type 2 diabetes*

Erythrocyte n-6 fatty acids		Multivariable-adjusted RRs (95% CIs)				
		Q1	Q2	Q3	Q4	*P* for trend
LA (C18:2n6)	Median (%)	8.11	9.43	10.29	11.41	
	Number of case subjects/total participants	72/682	73/683	65/683	66/683	
	Model 1	1.00 (Reference)	0.98 (0.73, 1.33)	0.94 (0.69, 1.28)	0.97 (0.71, 1.32)	0.77
	Model 2	1.00 (Reference)	1.00 (0.74, 1.35)	0.95 (0.70, 1.31)	0.98 (0.72, 1.33)	0.81
	Model 3	1.00 (Reference)	0.93 (0.69, 1.24)	0.93 (0.69, 1.26)	0.91 (0.67, 1.24)	0.59
GLA (γC18:3n6)	Median (%)	0.02	0.03	0.04	0.07	
	Number of case subjects/total participants	44/682	61/683	78/683	94/683	
	Model 1	1.00 (Reference)	1.33 (0.92, 1.92)	1.59 (1.12, 2.26)	1.88 (1.34, 2.64)	<0.001
	Model 2	1.00 (Reference)	1.35 (0.94, 1.95)	1.59 (1.11, 2.25)	1.85 (1.31, 2.61)	<0.001
	Model 3	1.00 (Reference)	1.22 (0.85, 1.74)	1.43 (1.01, 2.03)	1.72 (1.21, 2.44)	<0.001
AA (C20:4n6)	Median (%)	7.73	10.90	12.02	13.41	
	Number of case subjects/total participants	77/682	75/683	68/683	56/683	
	Model 1	1.00 (Reference)	0.87 (0.64, 1.17)	0.84 (0.62, 1.14)	0.81 (0.59, 1.12)	0.19
	Model 2	1.00 (Reference)	0.86 (0.64, 1.17)	0.84 (0.62, 1.14)	0.81 (0.59, 1.12)	0.21
	Model 3	1.00 (Reference)	0.89 (0.65, 1.22)	0.96 (0.69, 1.35)	1.00 (0.71, 1.40)	0.85
Total n-6 PUFAs	Median (%)	16.76	20.77	22.32	23.97	
	Number of case subjects/total participants	71/682	81/683	63/683	61/683	
	Model 1	1.00 (Reference)	1.05 (0.78, 1.41)	0.90 (0.66, 1.24)	0.97 (0.70, 1.33)	0.60
	Model 2	1.00 (Reference)	1.04 (0.77, 1.41)	0.92 (0.67, 1.26)	0.96 (0.69, 1.32)	0.60
	Model 3	1.00 (Reference)	1.02 (0.75, 1.39)	0.98 (0.70, 1.36)	1.05 (0.76, 1.47)	0.83

*Multivariable-adjusted RRs (95% CIs) were calculated for Q2–Q4 of the erythrocyte n-6 fatty acids using Q1 as the reference group using Poisson regression models. Covariates included in model 1 were age, sex, BMI, and WHR; model 2, model 1 plus education, household income, smoking and alcohol drinking status, physical activity, total energy intake, and family history of diabetes; and model 3 as model 2 plus baseline erythrocyte total n-3 PUFAs and fasting glucose. *P* value for trend was calculated based on per-quartile increase in the corresponding PUFA.

For β-diversity, GLA and AA significantly ($P < 0.01$) contributed to dissimilarities in gut microbiota composition at the genus level (Supplementary Table 14). Specifically for GLA, the gut microbiota composition at genus level was different across quartile groups of GLA concentrations (Supplementary Table 15), with $P = 0.006$ for comparison between Q1 and Q4, as visualized in Fig. 1*C*, for example.

Cross-Sectional Association of Gut Microbiota Diversity With Type 2 Diabetes

Patients with type 2 diabetes had a lower α-diversity represented by observed OTUs, Shannon diversity index, Simpson index, and Chao index compared with participants without type 2 diabetes (Fig. 1*B* and Supplementary Table 16). We also found that the overall β-diversity was different between participants with and without type 2 diabetes (Fig. 1*D* and Supplementary Table 17).

Taxonomic Profiles of Gut Microbial Community in Different Levels of GLA

We then focused on exploring gut genera potentially underlying the association between GLA and incident type 2 diabetes. At the genus level, the gut microbiota of participants within Q1 and Q4 of GLA was both dominated by *Bacteroides*, while the relative abundance of *Bacteroides* was significantly higher in the Q4 group ($P = 0.016$). We also identified *Rothia*, *[Eubacterium]*, and *Coprococcus* enriched in participants with high GLA levels (Q4) using the LDA method (Fig. 1*E*). We found several overlapping taxonomic biomarkers enriched in participants without type 2 diabetes and with low GLA level, including *Butyrivibrio*, *Blautia*, *Oscillospira*, *Odoribacter*, *S24-7 other*, *Rikenellaceae other*, and *Clostridiales other* (Fig. 1*E*). Genus *[Eubacterium]*, *S24-7 other*, *Blautia*, *Oscillospira*, *Odoribacter*, *Rikenellaceae other*, *Coriobacteriaceae other*, *Faecalibacterium*, and *Christensenellaceae other* were negatively correlated with triglycerides (Bonferroni-corrected $P < 0.05$) (Fig. 2). No correlation was observed for other metabolic trait–microbiota pairs (Supplementary Table 18).

Association of Dietary n-6 PUFAs With Incident Type 2 Diabetes and Gut Microbiota Diversity

Dietary LA was positively associated with incident type 2 diabetes (Q4 vs. Q1: RR 1.51; 95% CI 1.09, 2.09) (Supplementary Table 19). Dietary LA intake was associated with α-diversity (Chao index), but not β-diversity (Supplementary Tables 20 and 21).

CONCLUSIONS

In the present community-based prospective cohort of a Chinese population, we found that baseline erythrocyte GLA levels were positively associated with incident type 2 diabetes, independent of BMI and other potential confounders.

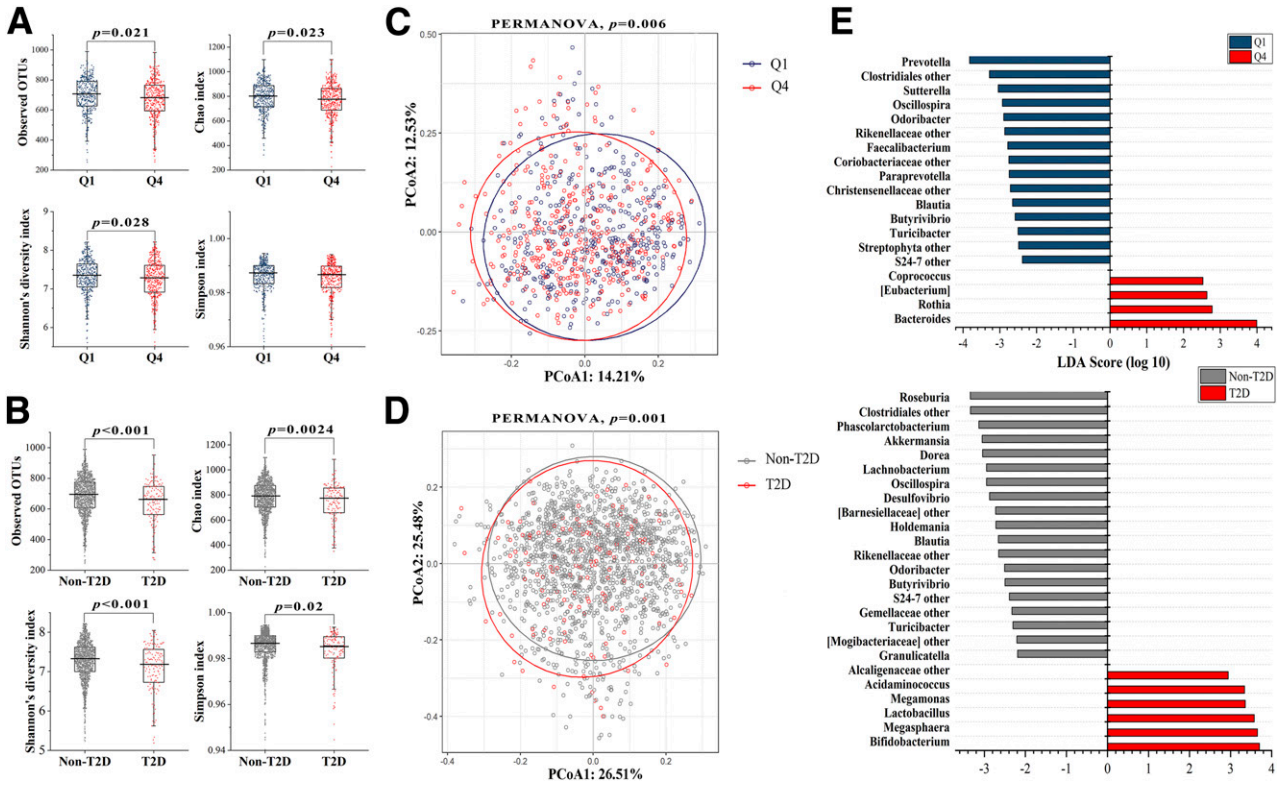

Figure 1—Erythrocyte GLA and gut microbiota covary in the development of type 2 diabetes risk. *A*: Community richness (observed OTUs and Chao index) and community diversity (Shannon diversity index and Simpson index) between Q1 and Q4 of erythrocyte GLA. The box plots feature the median (center line), upper and lower quartiles (box limits), and 1.5 times the interquartile range (whiskers). *P* values were calculated for Q4 of the erythrocyte n-6 fatty acids using Q1 as the reference group using a linear mixed model. *B*: Community richness (observed OTUs and Chao index) and community diversity (Shannon diversity index and Simpson index) across type 2 diabetes status. *P* values were calculated using a logistic regression model. *C*: Dissimilarities in gut microbiota composition between Q1 and Q4 of erythrocyte GLA represented by unconstrained principal coordinate analysis (PCoA) with the Bray Curtis dissimilarity index. Difference of erythrocyte GLA explained 0.3% of the dissimilarities in gut microbiota composition (PERMANOVA, $P = 0.006$). *D*: Dissimilarities in gut microbiota composition across type 2 diabetes status. Type 2 diabetes explained 0.25% of the dissimilarities in gut microbiota composition (PERMANOVA, $P = 0.001$). *E*: Gut microbial taxonomic biomarkers identified by the LDA at Q1 and Q4 of erythrocyte GLA and by type 2 diabetes status. T2D, patients with type 2 diabetes; Non-T2D, participants without type 2 diabetes.

Proportions of LA, AA, or total n-6 PUFAs were not associated with type 2 diabetes incidence. Our further investigation integrating gut microbiota data revealed that GLA may be associated with type 2 diabetes risk through a mechanism that varies diversity and composition of gut microbiota. Our findings suggest that n-6 PUFAs and gut microbiota covary in the development of type 2 diabetes risk, highlighting the presence of a novel mechanism of how fatty acids or gut microbiota influence type 2 diabetes risk. All of the results support our hypothesis about the interrelationships among n-6 PUFAs, gut microbiota, and type 2 diabetes.

Our findings suggest that high circulating GLA might be a risk factor for type 2 diabetes, which is consistent with several prospective studies in western countries (21,22). The null finding for AA in our present study is also consistent with those from previous studies (8). The findings for LA were inconsistent between our study and previous ones (8). Circulating LA reflects habitual consumption of n-6 PUFA, typically from plant-derived oils. The inconsistency may reflect that European and Chinese populations use plant oils differently with a combination of different foods. For instance, Chinese adults may consume plant oils with meat; therefore, the positive and negative effects of those would lead to the null finding for circulating LA. By contrast, an American or European population may use plant oils with vegetables or in a healthy diet pattern. Such dietary confounding might influence GLA findings little, as GLA is a product of tightly regulated desaturation of LA, and result in the consistent findings between studies.

Dietary LA showed positive association with type 2 diabetes, which was consistent with the results of circulating GLA. This is of interest, as there is no direct correlation between dietary LA and circulating GLA. Circulating GLA is derived from the metabolism of LA, and the LA was absorbed from the diet and was an established biomarker of dietary LA (23,24). Therefore, circulating GLA is mainly determined by the metabolism in the n-6 PUFA pathway, but is also indirectly affected by dietary n-6 PUFA intake. These results highlight that both diet and metabolism of the n-6 PUFAs may have important roles in the etiology of type 2 diabetes. Taken together, these results suggest that both dietary n-6 PUFAs and circulating n-6 PUFAs (i.e., GLA) may be important factors to consider for the population-level risk assessment of type 2 diabetes.

Given the putative relationship between gut microbiome and type 2 diabetes, we hypothesized and confirmed that gut microbiome diversity may play a role linking n-6 PUFAs and type 2 diabetes and that n-6 PUFAs may prospectively affect gut microbiota composition. Our findings are in line with a randomized controlled trial in which α-diversity and *Blautia* were reduced while *Bacteroides*

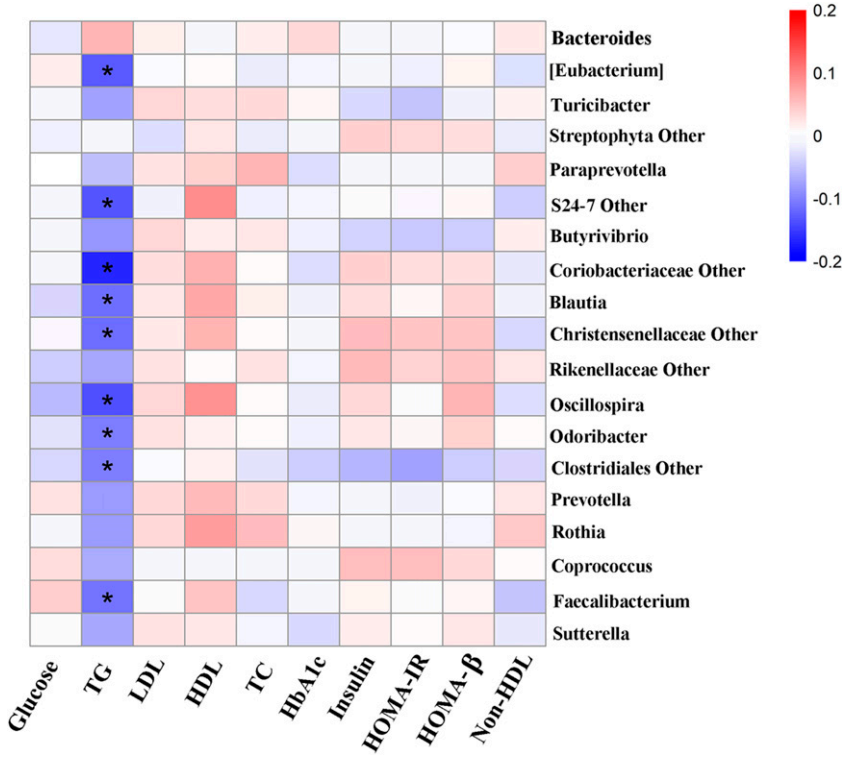

Figure 2—Heat map of the Spearman correlation coefficients between GLA-related microbes and 10 type 2 diabetes–related traits. The intensity of the colors represents the degree of association between GLA-related microbes and 10 type 2 diabetes–related traits as measured by the Spearman correlations. All significant correlations are marked with an asterisk (Bonferroni-corrected $P < 0.05$). HOMA-β, HOMA of β-cell function; HOMA-IR, HOMA of insulin resistance; TC, total cholesterol; TG, triglycerides.

was increased after higher-fat diet intervention (rich in n-6 PUFAs owing to exclusive use of soybean oil) (25). Although we did not observe higher relative abundance of *Bacteroides* in patients with type 2 diabetes in our study, *Bacteroides* has been found to be more abundant in type 2 diabetes in another Chinese population (10).

We found that *Butyrivibrio*, *Blautia*, *Oscillospira*, and *Odoribacter*, genera known to contain a series of butyrate-producing bacteria, are all enriched in participants within Q1 of GLA and participants without type 2 diabetes. These bacteria have been reported to have the ability to use fructose or glucuronate and were inversely associated with obesity (26–30). Moreover, in our study, *Blautia*, *Oscillospira*, and *Odoribacter* were inversely associated with circulating triglycerides, which were found to be positively associated with type 2 diabetes and higher level of GLA (31,32). Thus, our study suggests that lower levels of GLA may be correlated with factors increasing the abundance of these bacteria and preventing the development of type 2 diabetes.

The more detailed mechanism behind these associations remains unclear but may be related to chronic inflammation. Accumulating evidence supports the hypothesis that chronic low-grade inflammation is a risk factor for the development of type 2 diabetes (33) and that n-6 PUFA may play a vital role in the regulation of inflammation. GLA is produced from LA by the enzyme delta-6-desaturase and is further metabolized to dihomo-GLA (20:3n6), which undergoes oxidative metabolism by cyclooxygenases and lipoxygenases to produce anti-inflammatory eicosanoids, such as prostaglandins and leukotrienes (24). Moreover, imbalance of the gut microbiota and some specific microbes have been considered as contributors for inflammation by gut microbiota–derived lipopolysaccharide (LPS) (34). The LPS stimulates Toll-like receptors to influence innate immunity via its interaction with LPS receptors (i.e., CD14/Toll-like receptor 4 complex) (35). It can be proposed that LPS level is regulated by fat and fatty acids and associated with insulin resistance and type 2 diabetes (34,36). Moreover, the enrichment of inflammation-related bacteria (i.e., *Blautia*) (37) in participants with lower levels of GLA supports an important role of inflammation in the relationship among GLA, gut microbiota, and type 2 diabetes.

Other potential mechanisms linking n-6 PUFAs with gut microbiota and type 2 diabetes may be related to gut metabolites such as short-chain fatty acids and bile acids. As the most abundant bacterial fermentation products, short-chain fatty acids (such as acetate, propionate, and butyrate) played an essential role in the microbiota–host interaction. GLA was inversely associated with butyrate-producing bacteria in the current study. Depletion of butyrate contributed to the shift in the inflammatory status, leading to a dysbiosis of glucose metabolism (38). Besides, n-6 PUFAs could increase bile acid secretion from liver (39), which may act as an important signaling molecule linking n-6 PUFAs and gut microbiota. The generated bile acids could be metabolized to deoxycholic acid in the gut, which could disrupt hepatic endoplasmic reticulum stress, thereby impairing the glucose homeostasis (40). The above postulation about the potential mechanism linking n-6 PUFAs, gut microbiota, and type 2 diabetes may shed light on several new prevention targets for type 2 diabetes.

Strengths of the current study include the prospective cohort study design, which is rarely conducted in prior research on fatty acid–gut microbiota associations, and the collection and adjustment for a variety of information, including sociodemographic, lifestyle, and dietary factors, anthropometric parameters, and circulating blood biomarkers. In addition, fatty acid composition in erythrocytes reflects dietary fat intake over the past month or two, which may be superior (a more stable biomarker) than plasma or plasma phospholipid fraction (23). Several limitations merit attention. First, although we have adjusted for several potential confounders, due to the observational nature of the current study, we are not able to fully exclude the influence of residual confounding. Second, erythrocyte n-6 PUFAs were measured at baseline only and may not represent long-term status. Changes in dietary intake and levels of n-6 PUFA biomarkers over time are likely. Third, fecal samples were only collected during follow-up but not at

baseline. Repeated measure of both circulating n-6 PUFAs and gut microbiota or an intervention of n-6 PUFAs would enable us to characterize bidirectional effects of n-6 PUFA intakes on gut microbiota and of gut microbiota on circulating n-6 PUFAs. Nevertheless, our study fills the gap of the prospective associations between n-6 PUFA biomarkers and gut microbiota.

In conclusion, our study suggests that erythrocyte GLA biomarker is positively associated with incident type 2 diabetes in the Chinese population. Gut microbiota may play an important role in explaining the findings. The identification of GLA-related gut microbiota features may serve as a potential intervention target for further investigation. The detailed mechanism behind the association of GLA with type 2 diabetes risk and gut microbiota needs further clarification in the future.

Acknowledgments. The authors thank all of the participants and staff of the Guangzhou Nutrition and Health Study. The authors also thank the Westlake University Supercomputer Center for computational resources and related assistance.

Funding. This study was funded by the National Natural Science Foundation of China (81903316 and 81773416), Zhejiang Ten-thousand Talents Program (2019R52039), Westlake University (101396021801), and the 5010 Program for Clinical Researches of the Sun Yat-sen University (Guangzhou, China) (2007032). F.I. was funded by the U.K. Medical Research Council Epidemiology Unit core grant (MC_UU_12015/5).

The funders had no role in study design, data collection and analysis, decision to publish, or writing of the manuscript.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. Y.-m.C. and J.-S.Z. conceived the study concept and design. G.-d.C., F.-f.Z., H.-l.D., J.W., and C.X. collected the data. Z.M., J.-s.L., and Y.M. did the data analysis. Z.M., J.-s.L., Y.M., and J.-S.Z. wrote the first draft of the article. Z.J., M.S., W.G., Y.F., F.I., and Y.-m.C. contributed to the discussion and critical revision of the manuscript. All authors read, revised, and approved the final draft. Y.-m.C. and J.-S.Z. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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