



Immunometabolic profiling related with gestational diabetes mellitus: a nested case-control study of CD4⁺ T cell phenotypes and glycemetic traits

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Abstract

Aims To investigate immunometabolic associations of CD4⁺ T cell phenotypes with gestational diabetes mellitus (GDM).

Methods A nested case-control study was conducted comprising 53 pairs of GDM patients and matched controls within a prospective cohort. Metabolomic signatures related to both CD4⁺ T cell phenotypes and glycemetic traits among pregnant women were investigated by weighted gene co-expression network analysis (WGCNA). Multivariable-adjusted generalized linear models were used to explore the associations of CD4⁺ T cell phenotypes and selected metabolites with GDM. Mediation analysis was conducted to evaluate the mediating effect of selected metabolites on the relationship between CD4⁺ T cell phenotypes and glycemetic traits.

Results Higher levels of Treg cells (OR per SD increment (95%CI): 0.57 (0.34, 0.95), $p=0.031$) and increased expression of *Foxp3* (OR per SD increment (95%CI): 0.59 (0.35, 0.97), $p=0.039$) and *GATA3* (OR per SD increment (95%CI): 0.42 (0.25, 0.72), $p=0.002$) were correlated with a decreased risk of GDM. Plasma pyruvaldehyde, S-adenosylhomocysteine (SAH), bergapten, and 9-fluorenone mediated the association between Tregs and fasting plasma glucose (FPG), with mediation proportions of 46.9%, 39.6%, 52.4%, and 56.9%, respectively.

Conclusions Treg cells and *Foxp3* expressions were inversely associated with GDM risk, with potential metabolic mechanisms involving metabolites such as pyruvaldehyde and SAH.

Keywords CD4⁺ T cells · Metabolome · Gestational diabetes mellitus · Nested case-control study

Introduction

Gestational diabetes mellitus (GDM) refers to the glucose disorders with onset or first detection during pregnancy [1]. The International Diabetes Federation (IDF) estimated

that about 21.1 million live births, or 16.7%, were complicated by GDM in 2021 [2]. GDM leads to adverse pregnancy outcomes, including preterm delivery, macrosomia, and obstructed labor [3]. Furthermore, GDM affects the health of the mother and baby with possible long-term consequences. In particular, a history of GDM could lead to a 10-fold increased risk of developing type 2 diabetes mellitus (T2DM) for mothers and nearly 8-fold higher risk of pre-diabetes for their offspring [4, 5]. Therefore, research efforts to enhance our comprehension of GDM etiology and identify potential risk factors are of great clinical and public health significance.

Numerous maternal immune system adaptations are necessary during pregnancy to maintain immunological tolerance to the semi-allogeneic fetus. Prior studies suggested that GDM might result from a maternal endocrine disorder of adaptive immunity [6, 7]. In this case, immune

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system modulation should be essential to establish the optimal environment for GDM management. CD4⁺ T cells, as a major component of the adaptive immune response, can be further subdivided into T helper 1 cells (Th1 cells), Th2 cells, Th17 cells, and regulatory T cells (Treg cells), characterized by the expression of the transcription factor *T-bet*, *GATA3*, *ROR γ t*, and *Foxp3*, respectively [8]. Th1 and Th17 cells are proinflammatory and enhance the cytotoxic and phagocytic activity of macrophages, neutrophils, and natural killer cells by secreting IFN- γ or IL-17, respectively [8]. Conversely, Th2 and Tregs have demonstrated suppressive roles, which could boost the production or secretion of anti-inflammatory cytokines, such as IL-4, IL-5, IL-13, IL-10, and TGF β [8]. Several studies have observed an imbalance between proinflammatory and regulatory CD4⁺ subsets in GDM [9–11]. However, epidemiological studies of the associations between CD4⁺ T cells and the risk of GDM are still lacking, and the underlying mechanisms or mediators remain inadequately explored.

In the present study, 53 pairs of GDM patients and matched healthy pregnant controls were included to achieve the aims of (1) investigating the associations between CD4⁺ T cell phenotypes and GDM risk among pregnant women; (2) unveiling metabolic biomarkers related to pathways by which CD4⁺ T cell phenotypes modify GDM risks; (3) exploring whether and to what extent these metabolites could mediate the associations between CD4⁺ T cell phenotypes and glycemic traits.

Methods

Study design and population

This nested case-control study was conducted within a prospective cohort of pregnant women who attended their first antenatal care at the Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China, between October 2018 and March 2019. The details of this cohort have been described previously [12]. According to the inclusion and exclusion criteria, 1008 out of 1500 women were recruited at 10 to 15 weeks gestation. GDM was diagnosed at 24–28 weeks of gestation using an oral glucose tolerance test (OGTT) according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria in 2010, which were defined by three cut-offs: fasting plasma glucose (FPG), OGTT-1 h, or OGTT-2 h plasma glucose (PG) concentrations ≥ 5.1 , 10.0, or 8.5 mmol/L, respectively. By March 2019, 61 GDM patients diagnosed with IADPSG criteria were documented in the cohort. After excluding the patients with no peripheral blood mononuclear cells (PBMCs) samples ($n = 8$), 53 GDM patients were

included in the current analysis and one GDM was randomly matched with one non-GDM (1:1) with controlled age (± 2 years) and gestational weeks (± 2 weeks). This study was approved by the Research Committee of the Obstetrics and Gynecology Hospital of Fudan University (Ethics Approval number 2017-74) and followed the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Measurement of glycemic traits

In the second trimester of pregnancy (24–28 weeks of gestation), enrolled participants underwent physical examination and were provided 8 mL fasting blood samples.

Oral glucose tolerance tests (OGTTs) were performed in the morning after overnight fasting, with venous blood samples drawn before and after the ingestion of the glucose load in 60- and 120-min. Blood glucose measurements were performed using the electrochemical glucose oxidase method on an automatic biochemical analyzer (Hitachi 7600, Tokyo, Japan). The glucose area under the curve (AUC_{glucose}) on the OGTT was calculated by the trapezoidal rule. Hemoglobin A_{1c} (HbA_{1c}) was measured by VARIANT II TURBO HbA_{1c} Kit 2.0 (Bio-Rad Laboratories, Hercules, CA). Fasting insulin was measured by radioimmunoassay (Abbott Architect i2000, Illinois). HOMA-IR was estimated through the following formula: fasting glucose (mmol/L) \times fasting insulin (mIU/L)/22.5 [13].

Flow cytometry and cytokine measurements

A total of 4 mL whole blood samples from participants in the second trimester were collected in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were purified by density-gradient centrifugation using Ficoll-Hypaque (TBD, Tianjin, China).

For intracellular cytokine detection, freshly isolated PBMCs were washed with PBS supplemented containing 2.5% FBS (Sigma-Aldrich, St. Louis, MO, USA) and incubated with Fc receptor blocker (TrueStain fcX; Biolegend), culturing at 37 °C, 5% CO₂ (RPMI 1640 medium, Thermo) for 6 h of stimulation. PBMCs were stained at 4 °C for 25 min with PerCP/Cyanine5.5-anti-CD4 (Biolegend), and then cells were fixed with IC Fixation Buffer (eBioscience). After staining for membranal markers, intracellular labeling was performed for 45 min at room temperature: FITC-anti-IFN- γ (Biolegend), PE-anti-IL-4 (Biolegend), APC-anti-IL-17 A (Biolegend). For intracellular transcription factors detection, PBMCs were stained at 4 °C for 25 min with FITC-anti-CD3 (Biolegend) and PerCP/Cyanine5.5-anti-CD4 (Biolegend), and then cells were fixed with Foxp3 Fixation/Permeabilization buffers (eBioscience). And

then the following antibodies were added for incubation: PE-anti-Foxp3 (Biolegend), FITC- anti-T-bet (Biolegend), APC- anti-GATA3 (Biolegend), PE- anti-ROR γ T (BD Bioscience).

The immunophenotyping (Th1, Th2, Th17, and Treg) and corresponding transcription factors (*T-bet*, *GATA3*, *ROR γ t*, and *Foxp3*) of CD4⁺ T cells in PBMCs were performed by flow cytometry analyses with FACSCalibur (BD Biosciences, San Jose, CA).

Metabolomics analysis

In the sample preparation, 100 μ L of fasting plasma samples from the second trimester were mixed with 400 μ L methanol, incubated at -40 °C for 30 min to precipitate the proteins, and centrifuged at 15,000 rcf for 15 min at 4°C to collect the supernatant. Then, 400 μ L supernatant was dried under mild nitrogen, and the dry substance was redissolved in 50 μ L 50% methanol (including internal standards) to obtain the test sample for analysis. Aliquots of 5 μ L were taken from all treated samples, mixed in 1 tube, and used as the quality control (QC) sample in the analysis.

Nontargeted metabolomics testing was conducted using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-HRMS/MS). Chromatographic separation was performed on a ThermoFisher Ultimate 3000 UHPLC system with a Waters BEH C18 column (2.1 mm \times 100 mm, 1.7 μ m) with column temperature at 40 °C. The flow rate was 0.25 mL/min. For positive mode, the injection volume was 3 μ L. The mobile phases consisted of (A) water and (B) methanol, both with 0.1% formic acid. Linear gradient elution was performed with the following program: 0 min, 2% B; 12 min, 95% B; 15 min 100% B and held to 18 min; 18.1 min, 2% B and held to 20 min. For the negative mode, the injection volume was 3 μ L. The mobile phases consisted of (A) water and (B) methanol/water (95:5, v/v), both with 6.5mM ammonium bicarbonate. Linear gradient elution was performed with the following program: 0 min, 2% B; 9 min, 70% B; 14 min 100% B and held to 18 min; 18.1 min, 2% B and held to 20 min.

The mass spectrometer was operated in heated electrospray ionization positive (HESI+) and negative (HESI-) mode with a spray voltage of 3.5 kV. Both Capillary and Aux Gas Temperature were 350 °C. Simultaneously, the fragment ions information of top 10 precursors each scan was acquired by data-dependant acquisition (DDA) with HCD energy at 20, 30 and 40 eV, mass resolution of 17,500 FWHM, and AGC Target of 5×10^5 . Raw data of UHPLC-HRMS/MS were transformed to mzXML formatted by ProteoWizard and processed by XCMS (CAMERA) packages in R. The accurate m/z of precursors and product ions were matched against online databases including mzCloud,

Metlin, HMDB, and MassBank. Finally, 671 metabolites detected in more than 90% of the samples were included in the analysis. All data were normalized against total peak areas by log₁₀-transformed, and scaled for further analysis.

Co-abundance clustering of plasma untargeted metabolites

To generate co-abundance clusters, 671 metabolites detected in more than 90% of the samples were included. These metabolites were log₁₀-transformed and scaled, then clustered based on their co-abundance by the weighted gene co-expression network analysis (WGCNA) using the R package WGCNA (v.1.72-1). The following parameters were used for the analysis: soft thresholding $\beta = 12$, minimum cluster size = 5, deep split = 4, cut height = 0.999, and PAM clustering = F. The first principal component (PC1) of each co-abundance group (CAG) was calculated using the moduleEigengenes command and used as the representative value of the cluster for further analyses. The module membership (MM) was also evaluated to assess the importance of metabolites in each CAG.

Covariates

Upon enrollment, participants' general information was collected by questionnaire, including maternal age, pre-pregnancy body mass index (BMI), parity, physical activity, family history of diabetes, and socioeconomic status (SES). Pre-pregnancy BMI (kg/m²) was calculated by dividing pre-pregnancy weight in kilograms by the square of height in meters. Parity was categorized as 0 or ≥ 1 . Family history of diabetes was treated as dichotomized variables. SES is a comprehensive measure of economic and social standing based on income, education, and occupation [14]. The above indicators were collected from pregnant women and their husbands, then categorized and analyzed to determine three levels of SES: low, middle, and high. Participants were trained by clinical investigators to complete two 3-day food records (FRs) at 12~16 and 22~26 weeks of gestation to assess the total energy intake of pregnant women using the China Food Composition Database [15, 16]. Gestational weight gain (GWG) before OGTT was defined as the maternal weight difference between antenatal examination at 24~28 gestation weeks and before pregnancy.

Statistical analysis

Analyses were performed with R v.4.3.1 or SPSS 22. Statistical significance was set at a two-tailed *p* value < 0.05. Continuous data were presented as mean \pm standard deviation (normal distribution) or median (interquartile range)

(abnormal distribution). Categorical data were presented as n (%). For general statistical comparisons, t-tests or Mann-Whitney tests were used for continuous variables, and χ^2 tests were performed for categorical variables. The correlations of each CAG with CD4⁺ T cell phenotypes and glyce-mic traits (e.g., GDM status, FPG, OGTT-1 h PG, OGTT-2 h PG, AUC_{glucose}, insulin, and HOMA-IR) were assessed by partial Spearman's correlation (pSC) analysis, adjusting for maternal age, pre-pregnancy BMI, parity, SES, family history of diabetes, total energy intake, physical activity, and GWG before OGTT. All *p* values for multiple hypothesis testing were adjusted for multiple comparisons using the Benjamini-Hochberg method, and the false-discovery rate (FDR) adjusted *p* < 0.05 was considered statistically significant. Metabolites with MM > 0.80 in CAGs significantly associated with both CD4⁺ T cell phenotypes and GDM risk were selected for subsequent analyses.

The associations of CD4⁺ T cell phenotypes and selected metabolites with GDM were estimated using generalized linear models (GLMs) adjusting for potential covariates. The levels of CD4⁺ T cell phenotypes were natural log-transformed and standardized to z score (mean = 0 and standard deviation (SD) = 1) before analyses. To investigate whether the selected metabolites mediate the relationship

between CD4⁺ T cell phenotypes and glyce-mic traits, we performed mediation analysis using R package mediation (v.4.5.0). The bootstrapping method was applied, with coefficients estimated from 1000 simulations.

Results

Characteristics of the study participants

Characteristics of the 106 participants specified by GDM status are summarized in Table 1. Compared with controls, the GDM patients were more likely to have a higher BMI before pregnancy (*p* = 0.003) and have a family history of diabetes (*p* = 0.047). In the second trimester, levels of FPG (*p* < 0.001), OGTT-1 h PG (*p* < 0.001), OGTT-2 h PG (*p* < 0.001), AUC_{glucose} (*p* < 0.001), HbA_{1c} (*p* < 0.001), fast-ing insulin (*p* = 0.005), and HOMA-IR (*p* = 0.002) in GDM patients were significantly higher than those in the control. No difference was observed in age, parity, SES, moderate-intensity physical activity, total energy intake, and GWG before OGTT between women with GDM and without GDM.

Table 1 Demographic and clinical characteristics of study population by the GDM status

	Total (n = 106)	Non-GDM (n = 53)	GDM (n = 53)	<i>p</i>
Age (years)	31.0 (29.0, 33.0)	31.0 (29.0, 32.0)	32.0 (29.5, 33.5)	0.069
Pre-pregnancy BMI (kg/m ²)	21.3 ± 2.79	20.5 ± 2.04	22.1 ± 3.2	0.003
Parity				0.301
0	88 (83.0)	42 (79.2)	46 (86.8)	
≥ 1	18 (17.0)	11 (20.8)	7 (13.2)	
Socioeconomic status				0.404
low	33 (31.1)	15 (28.3)	18 (34.0)	
middle	56 (52.8)	27 (50.9)	29 (54.7)	
high	17 (16.0)	11 (20.8)	6 (11.3)	
Moderate-intensity physical activity (min/d)				0.401
< 10	100 (94.3)	49 (92.5)	51 (96.2)	
10~30	6 (5.7)	4 (7.5)	2 (3.8)	
Family history of diabetes	20(18.9)	6 (11.3)	14 (26.4)	0.047
Total energy intake (kcal/d)	2236.1 (1885.9,2529.4)	2245.8 (1906.1,2564.2)	2217.9 (1869.8,2483.2)	0.712
GWG before OGTT (kg)	8.7 ± 3.4	8.9 ± 2.8	8.6 ± 3.8	0.514
FPG (mmol/L)	4.3 (4.1, 4.7)	4.2 (4.0, 4.4)	4.6 (4.2, 5.1)	<0.001
OGTT-1 h PG (mmol/L)	8.5 (7.0, 10.2)	7.0 (5.7, 8.2)	10.2 (9.3,10.7)	<0.001
OGTT-2 h PG (mmol/L)	6.7 (5.7, 8.3)	5.8 (5.2, 6.5)	8.3 (7.5, 9.0)	<0.001
AUC for glucose	14.5 (11.9, 16.4)	12.0 (10.5, 13.6)	16.4 (15.5, 17.1)	<0.001
HbA _{1c} (%)	4.9 (4.7, 5.1)	4.8 (4.7, 5.0)	5.0 (4.9, 5.3)	<0.001
HbA _{1c} (mmol/mol)	30.0 (28.0, 32.0)	29.0 (28.0, 31.0)	31.0 (30.0, 34.0)	<0.001
Insulin (mmol/L)	9.2 (6.4, 12.7)	8.0 (5.6, 10.1)	10.8 (7.0, 15.7)	0.005
HOMA-IR	1.7 (1.1, 2.5)	1.5 (1.0, 2.0)	2.1 (1.3, 3.5)	0.002

Abbreviations GDM gestational diabetes mellitus, GWG gestational weight gain, OGTT oral glucose tolerance test, FPG fasting plasma glucose, PG plasma glucose, AUC for glucose the area under the glucose curve on the OGTT, HbA_{1c} hemoglobin A_{1c}, HOMA-IR the homeostatic model assessment for insulin resistance

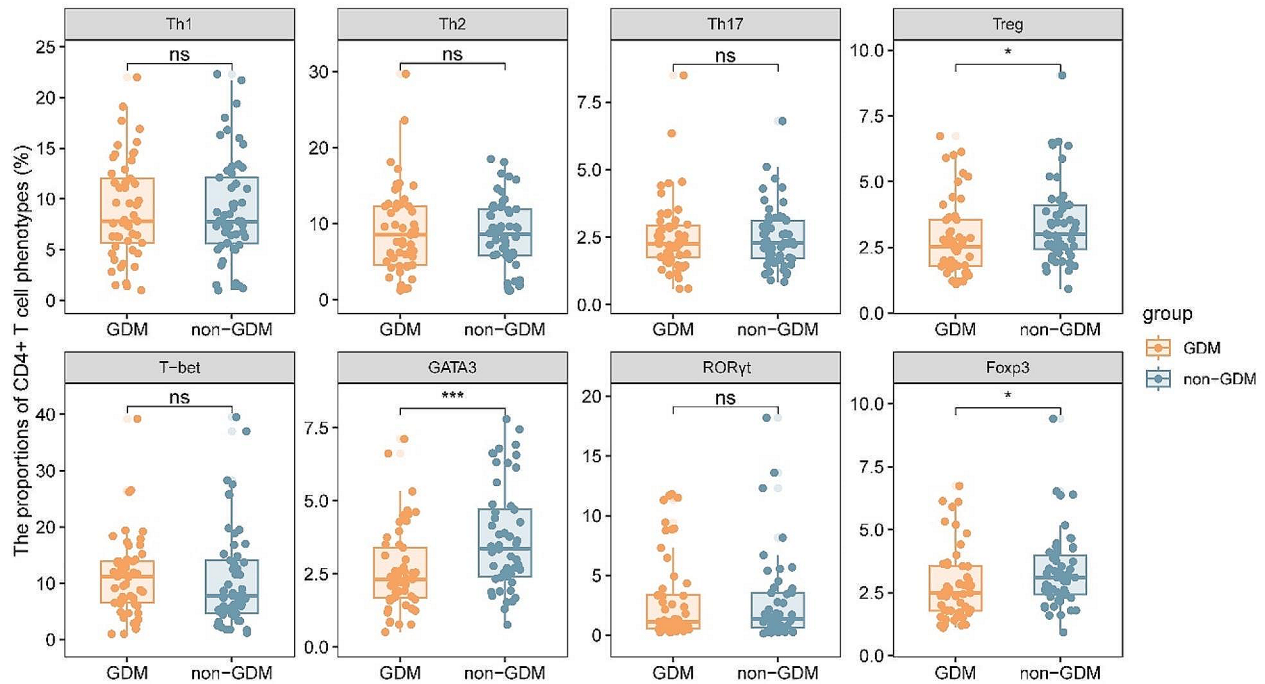


Fig. 1 The distributions of CD4⁺T cell phenotypes and transcription factors in GDM and non-GDM group

Table 2 Adjusted ORs (95% CI) for GDM per SD increment in CD4⁺T cell phenotypes*

CD4 ⁺ T cell phenotypes	OR per SD increment (95%CI)	<i>p</i>
Th1	1.14 (0.72, 1.79)	0.582
Th2	1.40 (0.84, 2.33)	0.196
Th17	1.02 (0.65, 1.60)	0.943
Treg	0.57 (0.34, 0.95)	0.031
<i>T-bet</i>	1.30 (0.84, 2.02)	0.244
<i>GATA3</i>	0.42 (0.25, 0.72)	0.002
<i>RORγt</i>	1.21 (0.77, 1.92)	0.408
<i>Foxp3</i>	0.59 (0.35, 0.97)	0.039

*Model was adjusted for maternal age, pre-pregnancy BMI, parity, socioeconomic status, family history of diabetes, total energy intake, physical activity, and GWG before OGTT

Association of CD4⁺T cell phenotypes with GDM risk

Comparative CD4⁺T cell phenotypes are shown in Fig. 1. Analysis of the CD4⁺ subsets revealed that women with GDM had significantly lesser percentages of Treg cells (median [interquartile range]: 2.5% [1.8 to 3.5] vs. 3.0% [2.4 to 4.1], $p=0.015$), and lower expression levels of *GATA3* (2.3% [1.7 to 3.5] vs. 3.4% [2.4 to 4.8], $p=0.001$) and *Foxp3* (2.5% [1.8 to 3.5] vs. 3.1% [2.4 to 4.0], $p=0.012$). Additional data are given in Online Resource 1:Table S1.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns means not significant.

Based on the adjusted generalized linear models, the increased percentage of Treg cells was related to a decreased risk of GDM (OR per SD increment (95%CI): 0.57 (0.34, 0.95), $p=0.031$; Table 2). The levels of *GATA3* (OR per SD increment (95%CI): 0.42 (0.25, 0.72), $p=0.002$; Table 2) and *Foxp3* (OR per SD increment (95%CI): 0.59 (0.35, 0.97), $p=0.039$; Table 2) also showed inverse associations with GDM.

Correlation of metabolite CAGs with CD4⁺T cell phenotypes and glycaemic traits

After co-abundance clustering, 41 metabolite CAGs were identified (Online Resource 1:Table S2). Adjusted for maternal age, pre-pregnancy BMI, parity, SES, family history of diabetes, total energy intake, physical activity, and GWG before OGTT, there were two CAGs significantly associated with Treg cells as well as the expression of *Foxp3* (Fig. 2a). Regarding glycaemic traits, the numbers of CAGs significantly associated with GDM status, FPG, OGTT-1 h PG, OGTT-2 h PG, AUC_{glucose}, insulin, and HOMA-IR were 3, 7, 7, 1, 7, 1, and 1, respectively (Fig. 2a).

Notably, we found that CAG 35 was simultaneously associated with Treg cells, *Foxp3*, and glycaemic traits, including GDM status, FPG, OGTT-1 h PG, and AUC_{glucose} (Fig. 2a). Therefore, the representative metabolites in CAG 35 were

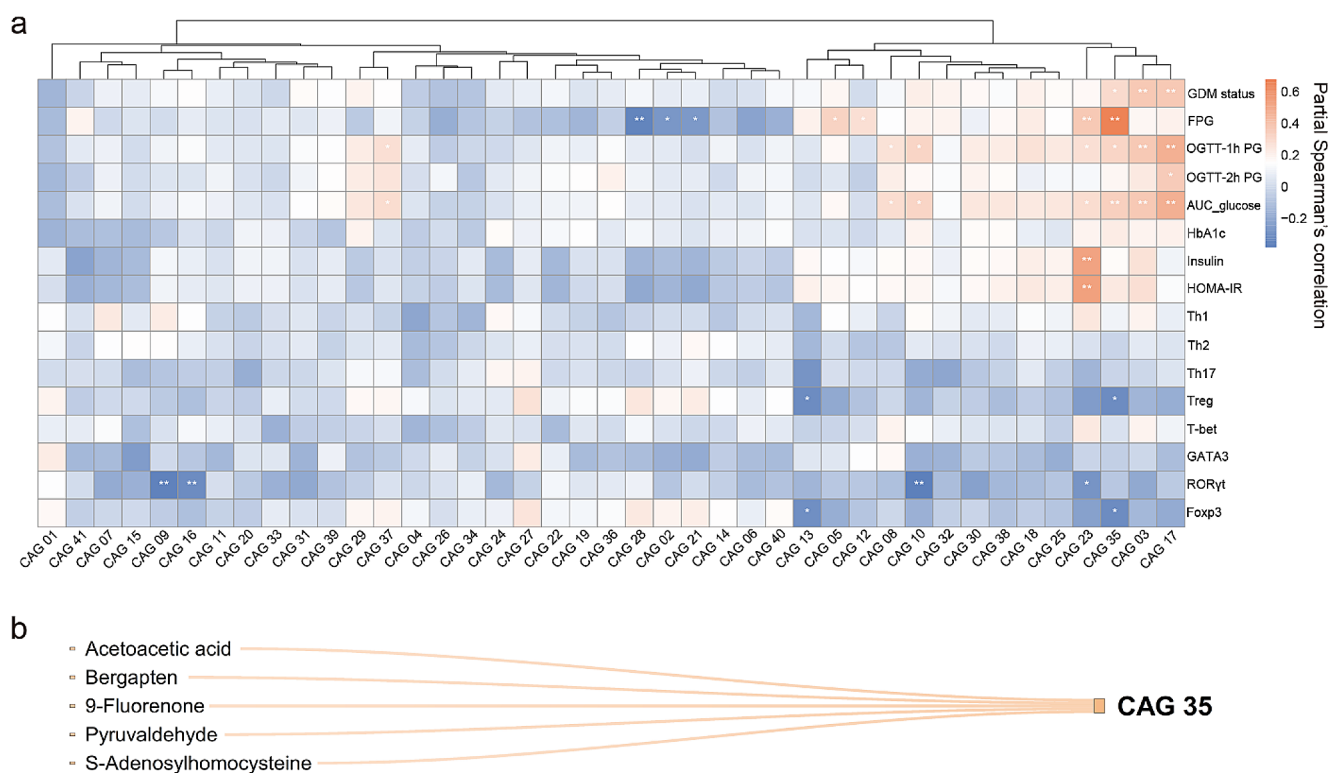


Fig. 2 The WGCNA of plasma metabolites, CD4⁺ T cell phenotypes, and glycemic traits among pregnant women **(a)** The partial Spearman's correlations of metabolite CAGs with CD4⁺ T cell phenotypes and glycemic traits in the second trimester. The *p* values were corrected by

the Benjamini-Hochberg procedure. * *P*_{adj} < 0.05, ** *P*_{adj} < 0.01. **(b)** Representative metabolites in CAG 35 related with both CD4⁺ T cell phenotypes and GDM risk

Table 3 Mediation of the association between CD4⁺ T cell phenotypes and glycemic traits through selected metabolites*

CD4 ⁺ T cell phenotypes	Mediator	Glycemic traits	Indirect effect, β (95% CI)	Direct effect, β (95% CI)	Total effect, β (95% CI)	%Mediated
Treg	Bergapten	GDM risk	-0.06 (-0.11, -0.01)	-0.05 (-0.15, 0.05)	-0.11 (-0.20, -0.01)	55.2%
Foxp3	Bergapten	GDM risk	-0.06 (-0.10, -0.02)	-0.04 (-0.14, 0.05)	-0.10 (-0.19, -0.01)	57.5%
Treg	Pyruvaldehyde	FPG	-0.08 (-0.15, -0.02)	-0.09 (-0.15, -0.03)	-0.17 (-0.25, -0.10)	46.9%
Treg	S-Adenosylhomocysteine	FPG	-0.07 (-0.12, -0.03)	-0.11 (-0.18, -0.03)	-0.17 (-0.26, -0.10)	39.6%
Treg	Bergapten	FPG	-0.09 (-0.17, -0.04)	-0.08 (-0.16, -0.01)	-0.17 (-0.25, -0.10)	52.4%
Treg	9-Fluorenone	FPG	-0.10 (-0.16, -0.05)	-0.08 (-0.14, -0.01)	-0.17 (-0.26, -0.10)	56.9%
Foxp3	Pyruvaldehyde	FPG	-0.08 (-0.15, -0.02)	-0.09 (-0.16, -0.02)	-0.17 (-0.26, -0.08)	48.4%
Foxp3	S-Adenosylhomocysteine	FPG	-0.07 (-0.12, -0.02)	-0.10 (-0.18, -0.03)	-0.17 (-0.25, -0.09)	38.9%
Foxp3	Bergapten	FPG	-0.09 (-0.16, -0.04)	-0.08 (-0.16, 0.00)	-0.17 (-0.25, -0.09)	53.5%
Foxp3	Pyruvaldehyde	HOMA-IR	-0.11 (-0.25, -0.02)	-0.15 (-0.36, 0.09)	-0.26 (-0.49, -0.02)	43.4%
Foxp3	Bergapten	HOMA-IR	-0.14 (-0.32, -0.02)	-0.12 (-0.31, 0.11)	-0.26 (-0.49, -0.03)	54.4%

* Negative mediating effect indicated that the indirect effect was in the opposite direction with the total effect. Mediation analyses were adjusted for maternal age, pre-pregnancy BMI, parity, socioeconomic status, family history of diabetes, total energy intake, physical activity, and GWG before OGTT. Results were expressed as β (95% CI) of CD4⁺ T cell phenotypes per SD increment

selected for further analyses (Fig. 2b). Additional data are presented in Online Resource 1: Table S3.

Mediation of metabolites between CD4⁺ T cell phenotypes and glycemic traits

Five metabolites in CAG 35, including acetoacetic acid, bergapten, 9-fluorenone, pyruvaldehyde, and

S-adenosylhomocysteine (SAH), served as the potential mediators due to their correlations with both CD4⁺ T cell phenotypes and GDM. Our results indicated that bergapten mediated the associations of Tregs and *Foxp3* with the risk of GDM, with a mediation proportion of 55.2% and 57.5%, respectively (Table 3). Pyruvaldehyde, SAH, bergapten, and 9-fluorenone mediated the relationship between Tregs and FPG, with mediation proportions of 46.9%, 39.6%, 52.4%,

and 56.9%, respectively (Table 3). Similarly, pyruvaldehyde, SAH, and bergapten mediated the correlation between *Foxp3* and FPG, with mediation proportions of 48.4%, 38.9%, and 53.5%, respectively (Table 3). Moreover, pyruvaldehyde and bergapten mediated the association between *Foxp3* and HOMA-IR, with mediation proportions of 43.4% and 54.4%, respectively (Table 3).

Discussion

In this nested case-control study on pregnant women with or without GDM, the proportion of Treg cells in PBMCs, and the levels of *Foxp3* and *GATA3* were found to be inversely associated with the risk of GDM. Based on the metabolome approach, we identified four plasma metabolites mediating the associations between Treg cells and glycemic traits, including pyruvaldehyde, SAH, bergapten, and 9-fluorenone.

In line with previous studies [9, 10], the Treg cell percentages and *Foxp3* expression were significantly reduced in women with GDM compared to non-GDM. For instance, Yang et al. [10] found that individuals with GDM had decreased levels of circulating Tregs and Treg-associated cytokines (TGF- β and IL-10) and elevated serum levels of proinflammatory cytokines (IL-6 and TNF- α) compared to those without GDM. Sheu et al. [11] also reported that GDM patients exhibited a higher ratio of Th1/Tregs and Th17/Tregs than women without GDM. In addition, the percentage of Tregs and its associated cytokine in the peripheral blood of T2DM patients was significantly lower than in a healthy population [17, 18]. In mouse models, adoptive transfer of Tregs could correct the reduced number of Tregs in insulin resistance and improve insulin sensitivity, confirming the protective effects of Tregs against glycemic dysregulation [19–21]. *GATA3*, as an anti-adipogenic transcription factor, has been identified as one of the potential targets for the impairment of adipogenesis and obesity-related metabolic dysfunction, including fatty liver disease, insulin resistance, and T2DM [22–24]. In our study, the expressions of *GATA3* showed an inverse relationship with GDM risk, which promoted *GATA3* as a potential therapeutic target for glycemic dysregulation during pregnancy. However, further research is needed to clarify the molecular effects of *GATA3*.

Additionally, our untargeted metabolomics analysis is the first to explore the metabolic potentials correlating the CD4⁺ T cell phenotypes and glucose regulations among pregnant women. In this study, we identified a series of plasma metabolites closely associated with both CD4⁺ T cell phenotypes and glycemic markers. Notably, mediation analysis revealed that pyruvaldehyde, SAH, bergapten, and 9-fluorenone might play mediating roles in the associations

between Tregs, *Foxp3*, and glycemic traits, which provided a novel perspective for studying the mechanism by which CD4⁺ T cell phenotypes impact glucose metabolism.

Pyruvaldehyde (2-oxopropanal or methylglyoxal) is predominantly produced as a byproduct of glycolysis during the degradation of triose phosphate intermediates, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) [25–27]. Our study demonstrated an association of pyruvaldehyde with diabetes risk, consistent with previous studies [28–30]. In young patients with uncomplicated type 1 diabetes mellitus (T1DM), serum pyruvaldehyde level was notably elevated compared to non-diabetic controls [29]. This finding has been recapitulated in newly diagnosed patients with T2DM [30], indicating a potential association between increased pyruvaldehyde levels and the onset of either T1DM or T2DM. Although the exact metabolic pathways whereby CD4⁺ T cells are involved in glucose homeostasis remain to be elucidated, the mechanisms underlying glucose dysregulation induced by pyruvaldehyde might involve the promotion of oxidative stress and inflammation through AGE/RAGE signaling [31], as well as the disruption of cellular responses to insulin, particularly affecting the ERK1/2 and AKT signaling pathway, which is crucial for the regulation of insulin sensitivity and glucose uptake [32, 33].

SAH is the precursor of homocysteine (Hcy) and is reversibly hydrolyzed by SAH hydrolase (SAHH) to homocysteine and adenosine [34]. Recent studies have suggested that elevated Hcy level represents an independent risk factor for cardiovascular disease [35], T2DM [36], and GDM [37]. Prior studies have also reported that erythrocytes or plasma SAH concentrations are significantly increased in patients with T2DM compared to nondiabetic patients [38]. Whereas studies of the relationship between SAH and GDM remain sparse, more prospective studies are needed to investigate the impacts of increased plasma SAH in pregnant women.

Moreover, recent studies demonstrated the antidiabetic properties of bergapten [39]. In contrast, our study found a positive correlation between bergapten and glucose dysregulation, mediating the associations of Tregs and *Foxp3* with the risk of GDM. Further research is needed to explore the heterogeneity and molecular mechanisms underlying these results.

To our knowledge, this is the first study to investigate the metabolic mediators between CD4⁺ T cell phenotypes and glycemic traits among pregnant women, which could enhance our comprehension of the intricate relationship between gestational adaptive immune responses and glucose metabolism and provide new strategies for early GDM prevention and glucose management during pregnancy. However, several limitations of our study merit discussion. First, this analysis was based on a nested case-control study

design with a relatively small sample size, making it challenging to explore the causality of the observed associations. Secondly, our participants were primarily residents of eastern China, which may limit the generalizability to other study populations. Thus, further prospective large-scale cohort and intervention studies are warranted to validate our findings.

Conclusions

An inverse relationship was observed between the levels of Treg cells and *Foxp3* expression in association with GDM risk, potentially mediated through metabolic pathways involving metabolites as pyruvaldehyde and SAH.

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Author contributions Conceptualization: Gengsheng He, Yuwei Liu; Methodology: Yuwei Liu, Xiaohui Wei; Data curation and investigation: Na Wang, Zhuo Sun, Zequn Deng; Validation: Wenyun Li, Tao Ying, Min Wu; Formal analysis, visualization and writing - original draft preparation: Xiaohui Wei; Writing - review and editing: Gengsheng He, Yuwei Liu, Xiaohui Wei; Funding acquisition: Gengsheng He, Yuwei Liu; Project administration: Gengsheng He, Na Wang; Supervision: Gengsheng He. All authors read and approved the final manuscript.

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Data availability Restrictions apply to the availability of all data analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will, on request, detail the restrictions and any conditions under which access to some data may be provided.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Committee of the Obstetrics and Gynecology Hospital of Fudan University (Ethics Approval number 2017-74).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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