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Postpartum defects in inflammatory response after gestational diabetes precede progression to type 2 diabetes: a nested case-control study within the SWIFT study

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ABSTRACT

Background: Gestational diabetes (GDM) is a distinctive form of diabetes that first presents in pregnancy. While most women return to normoglycemia after delivery, they are nearly ten times more likely to develop type 2 diabetes than women with uncomplicated pregnancies. Current prevention strategies remain limited due to our incomplete understanding of the early underpinnings of progression.

Aim: To comprehensively characterize the postpartum profiles of women shortly after a GDM pregnancy and identify key mechanisms responsible for the progression to overt type 2 diabetes using multi-dimensional approaches.

Methods: We conducted a nested case-control study of 200 women from the Study of Women, Infant Feeding and Type 2 Diabetes After GDM Pregnancy (SWIFT) to examine biochemical, proteomic, metabolomic, and lipidomic profiles at 6–9 weeks postpartum (baseline) after a GDM pregnancy. At baseline and annually up to two years, SWIFT administered research 2-hour 75-gram oral glucose tolerance tests. Women who developed incident type 2 diabetes within four years of delivery (incident case group, n = 100) were pair-matched by age, race, and pre-pregnancy body mass index to those who remained free of diabetes for at least 8 years (control group, n = 100). Correlation analyses were used to assess and integrate relationships across profiling platforms.

Results: At baseline, all 200 women were free of diabetes. The case group was more likely to present with dysglycemia (e.g., impaired fasting glucose levels, glucose tolerance, or both). We also detected differences between groups across all omic platforms. Notably, protein profiles revealed an underlying inflammatory response with perturbations in protease inhibitors, coagulation components, extracellular matrix components, and lipoproteins, whereas metabolite and lipid profiles implicated disturbances in amino acids and triglycerides at individual and class levels with future progression. We identified significant correlations between profile features and fasting plasma insulin levels, but not with fasting glucose levels. Additionally, specific cross-omic relationships, particularly among proteins and lipids, were accentuated or activated in the case group but not the control group.

Conclusions: Overall, we applied orthogonal, complementary profiling techniques to uncover an inflammatory response linked to elevated triglyceride levels shortly after a GDM pregnancy, which is more pronounced in women who progress to overt diabetes.

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1. Introduction

Pregnancy embodies a physiologic stress test for underlying metabolic dysfunction. It induces rapid metabolic adaptations such as increased fat mass [1], enhanced glucose production [2], and reduced insulin sensitivity [3,4], which are fine-tuned by the maternal system for the eventual return to the pre-pregnancy state following delivery [4,5]. Disruptions in these regulatory mechanisms may result in a new-onset hyperglycemic complication known as gestational diabetes mellitus (GDM) [6].

While GDM typically resolves following delivery, roughly 30 % of affected women reclassify as having prediabetes within three months postpartum [7,8]. GDM may therefore not only reveal latent susceptibilities [9] but also exacerbate long-term islet β-cell dysfunction, accelerating the onset of overt diabetes [10,11]. To curb this health burden, several landmark clinical trials for type 2 diabetes prevention following a GDM pregnancy were launched in the late 1990s. Promising reductions in progression to diabetes by thiazolidinediones were thwarted by concerns over troglitazone-induced hepatotoxicity [12,13] and pioglitazone use during breastfeeding [14,15]. Randomization to either metformin or intensive lifestyle changes effectively lowered incidence rates of diabetes over a 10-year follow-up [10]. Yet, women with a GDM history are nearly ten times more likely to develop type 2 diabetes than those with normoglycemic pregnancies [16,17], underscoring the gaps and cracks of current prevention measures.

Heterogeneity in responses to pharmacological and lifestyle intervention warrants a more comprehensive investigation into the early pathways for disease progression. The postpartum period facilitates a timely dissection into the early changes associated with a full recovery or progression to type 2 diabetes, which can be readily quantified in maternal plasma using omics. Applying these analytic methods may not only uncover novel biomarkers for high-risk subpopulations but also delineate their putative roles as effectors, protectors, or bystanders in key mechanisms. In fact, our prospective, longitudinal Study of Women, Infant Feeding, and Type 2 Diabetes after GDM Pregnancy (SWIFT) stands alone in employing large-scale, clinical metabolomic and lipidomic techniques to clarify the postpartum underpinnings of disease progression as well as the potential protective effect of lactation against diabetes development [7,18–22]. These clinical omic research studies characterized early metabolic disturbances after GDM pregnancy that precede the development of diabetes.

In the current nested case-control study, we now seek to expand our work on the SWIFT cohort using proteomics as it provides orthogonal and complementary insights into the early stages of progression to type 2 diabetes beyond the restricted purview of clinical observations and metabolomic investigation.

2. Materials & methods

2.1. Study design

SWIFT is an ongoing prospective, longitudinal epidemiologic research study of women after a GDM pregnancy. More specifically, 1035 pregnant women diagnosed with GDM (according to the Carpenter and Coustan criteria) who delivered at a Kaiser Permanente Northern California (KPNC) hospital were enrolled. All participants consented to three in-person research visits with 2-hour 75-gram oral glucose tolerance test (OGTT), anthropometry measurements, and clinical assessments, at 6–9 weeks postpartum (baseline) and annually thereafter for up to 2 years postpartum (follow-up).

The primary outcome of SWIFT is new-onset type 2 diabetes (diagnosed according to the American Diabetes Association criteria). Women who were positively indicated by the baseline 2-hour 75-gram OGTT results for diabetes underwent repeated testing on two separate occasions. A total of 1010 women returned to a non-diabetic state and were therefore included in the follow-up study for progression to diabetes. Post-baseline reclassification was performed based on two strategies: 1) annual research 2-hour 75-gram OGTTs at study visit for the first two years and 2) electronic health record surveillance (of laboratory testing, medical diagnoses from ICD codes, medication use) within the Kaiser Permanente integrated health system, which were reviewed every two years through October 2020 for the current analysis. Of the 990 women with follow-up testing for diabetes, 226 (23 %) developed new-onset type 2 diabetes. The SWIFT timeline is shown in Fig. 1A. Preliminary outcomes have been published elsewhere [7,23,24] and can also be found at ClinicalTrials.gov (#NCT01967030).

The present study employs a nested case-control design within the broader SWIFT cohort, as outlined in Fig. 1B. We included all women who progressed to type 2 diabetes within 4 years post-baseline (n = 127) and selected a control group of women who remained diabetes-free for at least 8 years. The final study population included a total of 100 cases and 100 controls, pair-matched by age, race, and pre-pregnancy body mass index.

2.2. Plasma handling & biochemical analysis

All profiling techniques were performed on EDTA-treated plasma samples collected during the fasting timepoint of the 2-hour 75-gram research OGTT at the baseline SWIFT research visit. Aliquots were stored at −80°C until further analyses. Biochemical assessment of baseline plasma samples was performed within several weeks of baseline visit (Northwest Lipid Research Laboratories, Seattle, Washington). All subsequent omic analyses were performed in singlicate.

2.3. Discovery proteomics

We manually prepared all samples in-house using a previously published workflow [25]. Briefly, plasma samples were boiled in a one-pot mixture for simultaneous lysis, reduction, and alkylation. Linearized proteins were then trypsinized overnight. Peptides were then loaded onto EvoTips and separated on a pre-set 60SPD gradient on an EvoSep One liquid chromatography system coupled to a Bruker timsTOF Pro mass spectrometer. Additionally, we prepared 10 technical replicates from a pooled sample and demonstrated high reproducibility (Fig. S1). Raw data files were analyzed on a closed search against the human UniProt FASTA database followed by label-free quantification with match-between-runs (LFQ-MBR) using a built-in FragPipe workflow [26]. Using the protein output lists from FragPipe, we removed non-human contaminants, log2-transformed total MaxLFQ intensities, and applied a filter cut-off of 30 % missingness. Batch correction to minimize plate effects and imputation to handle missing values were performed.

2.4. Targeted metabolomics

We accessed a metabolomic dataset from a previously published SWIFT study that measured 188 metabolites from 658 women who had GDM [18], including 200 women from the present study. Metabolite concentrations were measured with Biocrates AbsoluteIDQ p180 plates, according to the manufacturer's instructions, using a targeted mass spectrometry-based technique known as multiple reaction monitoring. Concentrations below the quantitative threshold (either the limit of detection or lower limit of quantification) were deemed missing and subsequently replaced with half of its quantitative threshold value for each specific analyte. A filter cut-off of 30 % missingness was applied to isolate the 128 robustly quantified proteins for further analyses.

2.5. Targeted lipidomics

We accessed a lipidomic dataset from a previously published nested case-control SWIFT study that measured 1008 lipid species from 350 women who had gestational diabetes [20], including 100 cases and 54 controls from the present study. Therefore, for the lipidomic analyses,

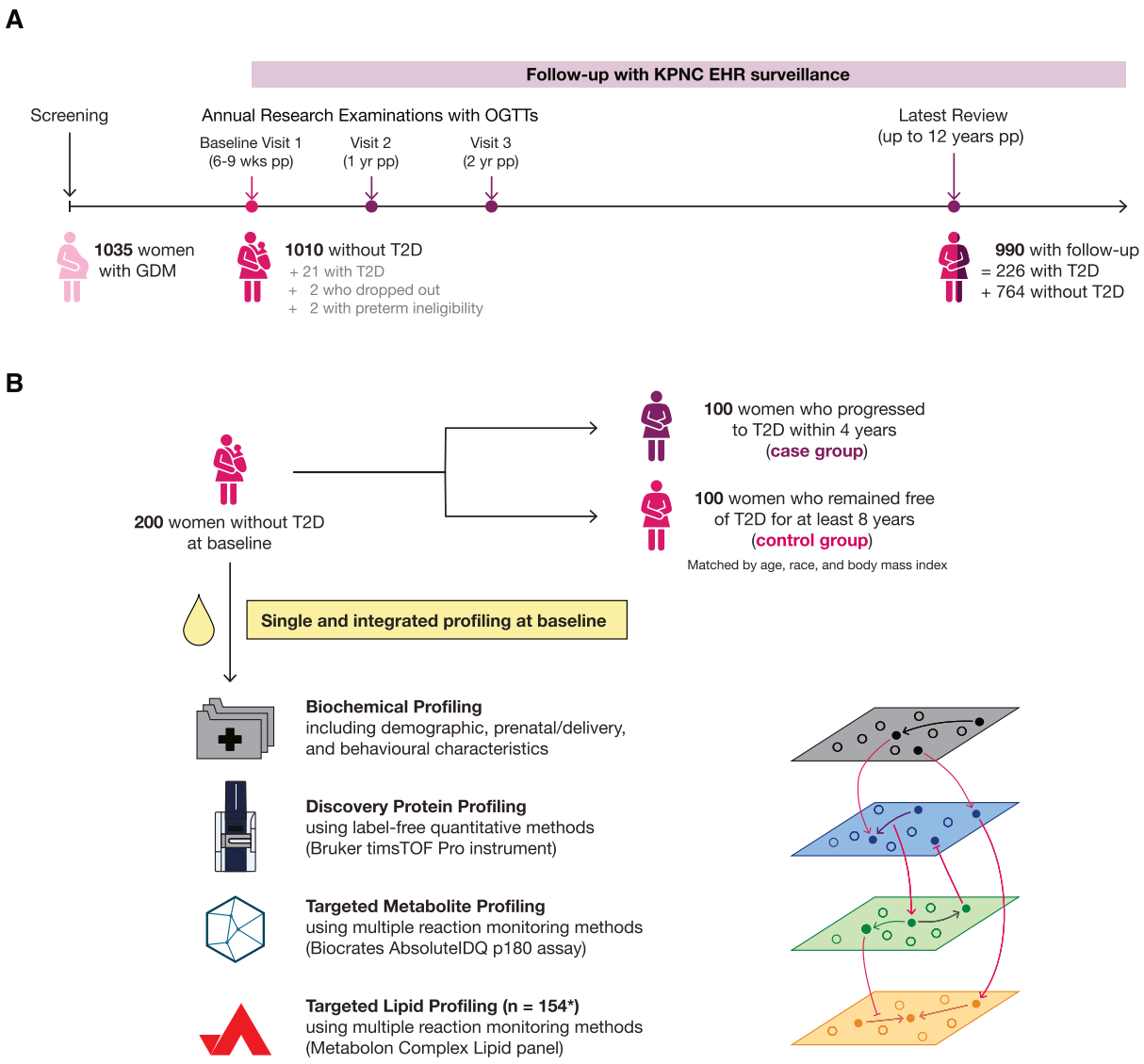

Fig. 1. Overview of the study design. (A) The Study of Women, Infant Feeding and Type 2 Diabetes After GDM Pregnancy (SWIFT) recruited 1035 pregnant women with GDM. All participants consented to three annual in-person research examinations, including 2-hour 75-gram oral glucose tolerance tests (OGTTs), anthropometry measurements, and clinical assessments, at baseline (6–9 weeks postpartum, pp) and for the first two years after delivery. Ongoing follow-up through the Kaiser Permanente Northern California (KPNC) electronic health records (EHR) system captured clinical assessments and new diagnoses of diabetes. (B) Current nested case-control study within the SWIFT cohort on 100 women who progressed to T2D within 4 years and 100 peers who remained free of diabetes for at least 8 years. Baseline plasma profiles were examined to identify potential networks of proteins, metabolites, and lipids associated with the progression to T2D using an integrative approach.
*Lipidomic data was not available for 46 women in the control group.

we profiled a subset of the present study's full cohort (Table S1). These 1008 lipid species were measured and characterized by fatty acid composition with the Metabolon Complex Lipid Panel using multiple reaction monitoring technology. We applied a stringent filter cut-off of 100 % quantitation (accounting for a reduced sample size) to isolate the 739 robustly quantified proteins for further differential analyses.

2.6. Multi-omic integration

Joint pathway analysis was first conducted to simultaneously evaluate the standalone protein list and the combined metabolite/lipid list using MetaboAnalyst (version 4.0) [27]. We also examined co-expression networks of differentially abundant metabolites/lipids and their protein correlates based on Spearman's rank correlation analyses of overall and within-group relationships. Significant correlations were defined by Benjamini-Hochberg (BH) adjustment ($q < 0.05$) and absolute Spearman's rank correlation coefficient cut-offs ($|r_s| > 0.3$ or 0.4 for full-cohort and within-group analyses, respectively).

Full details on the *SWIFT design, Matching criteria, Plasma protein profiling, Differential analysis of proteins, metabolites, and lipids, Bioinformatics* and other analyses can be found in the Supplemental Methods.

3. Results

3.1. Clinical characterization of study cohort

We conducted a nested case-control study on a diverse subsample of 200 women following a GDM pregnancy from the SWIFT cohort (aged 33.9 ± 4.6 years; 13 % White, 37 % Asian, 10 % Black, and 40 % Hispanic; and 57 % obese at study baseline) (Table 1, Fig. 1). Women who developed incident type 2 diabetes within four years of delivery (incident case group, n = 100) were pair-matched by age, race, and pre-

Table 1
Characteristics of the cohort by future outcome.

Demographic & clinical characteristics	Incident T2D (n = 100)	No T2D (n = 100)	*P*
Mean age at baseline, years (SD)	34.1 (4.7)	34.0 (4.5)	0.8611
Race, n			1.0000
Non-Hispanic White	13	13	
Asian	37	37	
Non-Hispanic Black	10	10	
Hispanic	40	40	
Family history of diabetes, n	62	45	0.0233
Personal history of PCOS, n	14	8	0.2585
Current history of smoking, n	1	2	0.3650

Anthromopetric characteristics	Incident T2D	No T2D	*P*
Median pre-pregnancy BMI, kg/m^2 (IQR)	30.2 (26.8–34.3)	30.2 (26.4–33.8)	0.8883
Pre-pregnancy BMI categories, *n*			0.9772
Under/normal weight (<25 kg/m^2)	17	16	
Overweight (25 to 30 kg/m^2)	31	32	
Obese (>30 kg/m^2)	52	52	
Median post-pregnancy BMI, kg/m^2 (IQR)	30.9 (27.8–34.7)	30.3 (27.5–34.1)	0.5951
Post-pregnancy BMI categories, n			0.5905
Under/normal weight (<25 kg/m^2)	12	9	
Overweight (25 to 30 kg/m^2)	30	36	
Obese (>30 kg/m^2)	58	55	
Mean weight, kg (SD)	80 (18)	79 (17)	0.7596
Mean height, cm (SD)	159 (7)	159 (7)	0.8942
Mean waist circumference, cm (SD)	94 (12)	92 (13)	0.3281
Mean weight loss after pregnancy, kg (IQR)	8 (4)	9 (4)	0.2281
Mean body fat, % (SD)	47 (6)	47 (7)	0.9442

Prenatal & delivery characteristics	Incident T2D	No T2D	*P*
GDM treatment, n			0.0006
Diet	46	70	
Oral medication only	45	29	
Insulin	9	1	
Median sum of 4 prenatal 3-hour 100-g OGTT glucose z-scores for GDM diagnosis (IQR)[a]	1.5 (−0.6–3.4)	−0.8 (−2.0–1.0)	<0.0001
Caesarean section delivery, n	39	32	0.3753
Parity, n			0.8275
Primiparous (1 birth)	32	35	
Biparous (2 births)	37	33	
Multiparous (>2 births)	31	32	

Biochemical characteristics at study baseline (6-9 weeks postpartum)	Incident T2D	No T2D	*P*
Median fasting plasma glucose (IQR)			<0.0001
mmol/L	5.6 (5.3–6.1)	5.1 (4.8–5.4)	
mg/dL	102 (96–111)	93 (89–98)	
Median 2-hour post-load plasma glucose (IQR)			<0.0001
mmol/L	7.1 (6.3–8.4)	5.8 (4.9–7.0)	
mg/dL	129 (115–153)	105 (89–127)	
Median fasting plasma insulin (IQR)			<0.0001
pmol/L	149 (104–225)	113 (80–154)	
μU/mL	24 (17–38)	19 (13−21)	
Median 2-hour plasma insulin (IQR)			<0.0001
pmol/L	672 (476–1004)	478 (343–661)	
μU/mL	112 (79–167)	81 (57–111)	
Median HOMA-IR score (IQR)	6.5 (4.3–9.4)	4.2 (3.0–5.8)	<0.0001
Median HOMA-B score (IQR)	235 (169–334)	241 (190–334)	0.8546
Median $ISI_{0,120}$ score (IQR)	1.2 (1.1–1.5)	1.6 (1.4–1.8)	<0.0001
Glycemic tolerance categories, *n*			<0.0001
Normal	31	74	
IFG only	32	14	
IGT only	13	10	
Both IFG and IGT	24	2	
Mean fasting plasma HDL-C (SD)			0.3116
mmol/L	1.3 (0.3)	1.3 (0.3)	
mg/dL	49 (13)	51 (12)	
Mean fasting plasma LDL-C (SD)			0.5253
mmol/L	3.2 (0.8)	3.2 (0.8)	
mg/dL	122 (31)	125 (31)	
Mean fasting plasma total cholesterol (SD)			0.9839

(*continued on next page*)

Table 1 (*continued*)

Biochemical characteristics at study baseline (6-9 weeks postpartum)	Incident T2D	No T2D	*P*
mmol/L	5.2 (0.9)	5.2 (0.9)	
mg/dL	201 (34)	202 (36)	
Median fasting plasma triglycerides (IQR)			0.0351
mmol/L	1.3 (0.9–2.2)	1.1 (0.8–1.6)	
mg/dL	118 (77–195)	96 (73–140)	

Behavioral characteristics at study baseline (6-9 weeks postpartum)	Incident T2D	No T2D	*P*
Median dietary glycemic index (IQR)	232 (170–312)	211 (167–279)	0.1761
Median dietary fiber, g/100 kcal (IQR)	1.0 (0.8–1.2)	0.9 (0.7–1.2)	0.1573
Mean dietary animal fat, % of kilocalories (SD)	26.8 (7.3)	26.0 (8.8)	0.4946
Median total physical activity, metabolic equivalent hours per week (IQR)	46.8 (36.0–71.9)	44.2 (33.3–58.9)	0.0777
Lactation intensity categories, n			0.2056
Exclusively breastfeeding	18	16	
Mostly breastfeeding	35	49	
Mostly formula-feeding	22	14	
Exclusively formula-feeding or <3 weeks breastfeeding	25	21	
Median lactation duration through 2 months postpartum (IQR)	1.7 (0.6–2.0)	1.9 (1.0–2.0)	0.4396

Case and control groups were matched by age, race, and pre-pregnancy BMI in this case-control study nested within the broader SWIFT population of 1010 women who did not have diabetes at baseline (6–9 weeks postpartum) after GDM pregnancy. Categorical variables were compared using the chi-squared test; continuous variables were assessed using the two-sample independent *t*-tests for normally distributed variables (presented as mean and SD) or Mann-Whitney tests for non-normally distributed variables (presented as median and IQR). Patient data were incomplete for body fat percentage (for 14 women in the case group and 23 in the control group) and waist circumference (2 in the case group and 1 in the control group); dietary characteristics ([i.e., fiber, glycemic index, and percentage of animal fat in diet] 1 in the control group); and physical activity (1 in the control group).

BMI, body mass index; DM, diabetes mellitus; GDM, gestational diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HOMA-B, homeostatic metabolic assessment of beta-cell function; HOMA-IR, homeostatic metabolic assessment of Insulin Resistance; IFG, impaired fasting glucose levels; IGT, impaired glucose tolerance; IQR, interquartile range; $ISI_{0,120}$, insulin sensitivity index, LDL-C, low-density lipoprotein cholesterol; OGTT, oral glucose tolerance test; PCOS, polycystic ovarian syndrome; SD, standard deviation.

[a] Sum of the four z-scores for the prenatal 100-g OGTT glucose values (fasting, 1, 2, and 3 h timepoints).

pregnancy body mass index to those who remained free of diabetes for at least 8 years (control group, n = 100). Median time-to-diabetes in the case group was 1.7 years (interquartile range, IQR, from 0.9 to 2.2).

Comprehensive participant characteristics are summarized in Table 1. Notably, we observed baseline differences in glycemic status between those who did and did not develop diabetes, as the incident case group was at least three times more likely to present with some form of baseline glucose abnormality such as impaired fasting glucose levels (as having fasting levels above 5.5 mmol/L or 100 mg/dL; 56 % vs. 16 %, $p < 0.01$), impaired glucose tolerance (as having 2-hour post-load levels in a 75-g oral glucose tolerance test above 7.8 mmol/L or 140 mg/dL; 37 % vs. 12 %, $p < 0.01$), or both (24 % vs. 2 %, $p < 0.01$), compared to the control group (Table 1). Altogether, only 31 % of the case group had normal glycemic control at baseline, compared to 74 % of those who did not develop diabetes ($p < 0.01$). Notable differences associated with baseline glycemic status were also reflected in prenatal history and baseline biochemistry. While the homeostatic model assessment scores for insulin resistance (HOMA-IR) were indeed higher in the case group, compared to the control group ($p < 0.01$), scores for beta cell function (HOMA-B) were similar between groups ($p = 0.85$). Expectedly, insulin sensitivity index ($ISI_{0,120}$), calculated from OGTT fasting and endpoint measurements [24], were markedly lower in the case group ($p < 0.01$). As a result, this early postpartum stage may be characterized by a dysglycemic state coupled with insulin resistance, possibly extant from before delivery, especially in women who later developed type 2 diabetes.

3.2. Plasma protein profile of the early postpartum period after GDM pregnancy

Given that the study baseline (6–9 weeks postpartum) remains a vastly unexplored timepoint in proteomics, we first sought to characterize the plasma protein profile of the postpartum period shortly after a GDM pregnancy. Using label-free discovery techniques, we quantified a total of 358 unique proteins (average of 235 per sample, Fig. S2A) spanning four orders of magnitude (Fig. S2B). Log-transformed intensities exhibited a bimodal relationship with reference blood concentrations from the Human Protein Atlas [28] (Fig. S2C): one group of proteins whose intensities were linearly correlated with reference concentrations (Spearman's rank correlation coefficient $r_s = 0.75$) and another group lacking any correlation ($r_s = -0.05$). After applying a filter cut-off of 30 % missingness, we observed that most of these latter proteins were removed and therefore deemed as unreliably quantified.

From the 358 total proteins remained a subset of 210 robustly quantified proteins. This subset included a higher percentage of proteins with some degree of tissue enrichment or enhancement, compared to the total proteome (Fig. S2D). Tissue expression analyses highlighted a preponderance of liver-associated proteins, accounting for 46 % of proteins (Fig. S2E). Given our interest in the postpartum profile, we noted that the placenta was the top enriched tissue within the reproductive system with seven mapped proteins, including the primariy hemoglobin in fetal circulation, HbG. Its quantification demonstrates that our plasma protein profile may contain potential remnants of pregnancy at 6–9 weeks postpartum. Protein enrichments to the placenta and/or endometrium may reflect physiologic processes underlying the return to the pre-pregnancy state such as involution. Accordingly, our protein profile therefore captures time-sensitive nuances of the female plasma profile.

3.3. Proteomic differences between groups

Of the 210 robustly quantified proteins, 21 were differentially abundant between women who did and did not develop new-onset type 2 diabetes within 4 years after baseline (*t*-test, $p < 0.05$, Fig. 2A, Table S2). Three proteins (proteoglycan-4, PRG4; pigment epithelium-derived growth factor, PEDF; and alpha-1-antichymotrypsin, AACT) remained significant after multiple testing correction (BH, $q < 0.1$), reflecting modest separability and discrimination of profiles between groups at this postpartum juncture (Fig. S3). To better capture the broader schemes of networks, we performed enrichment analyses on the

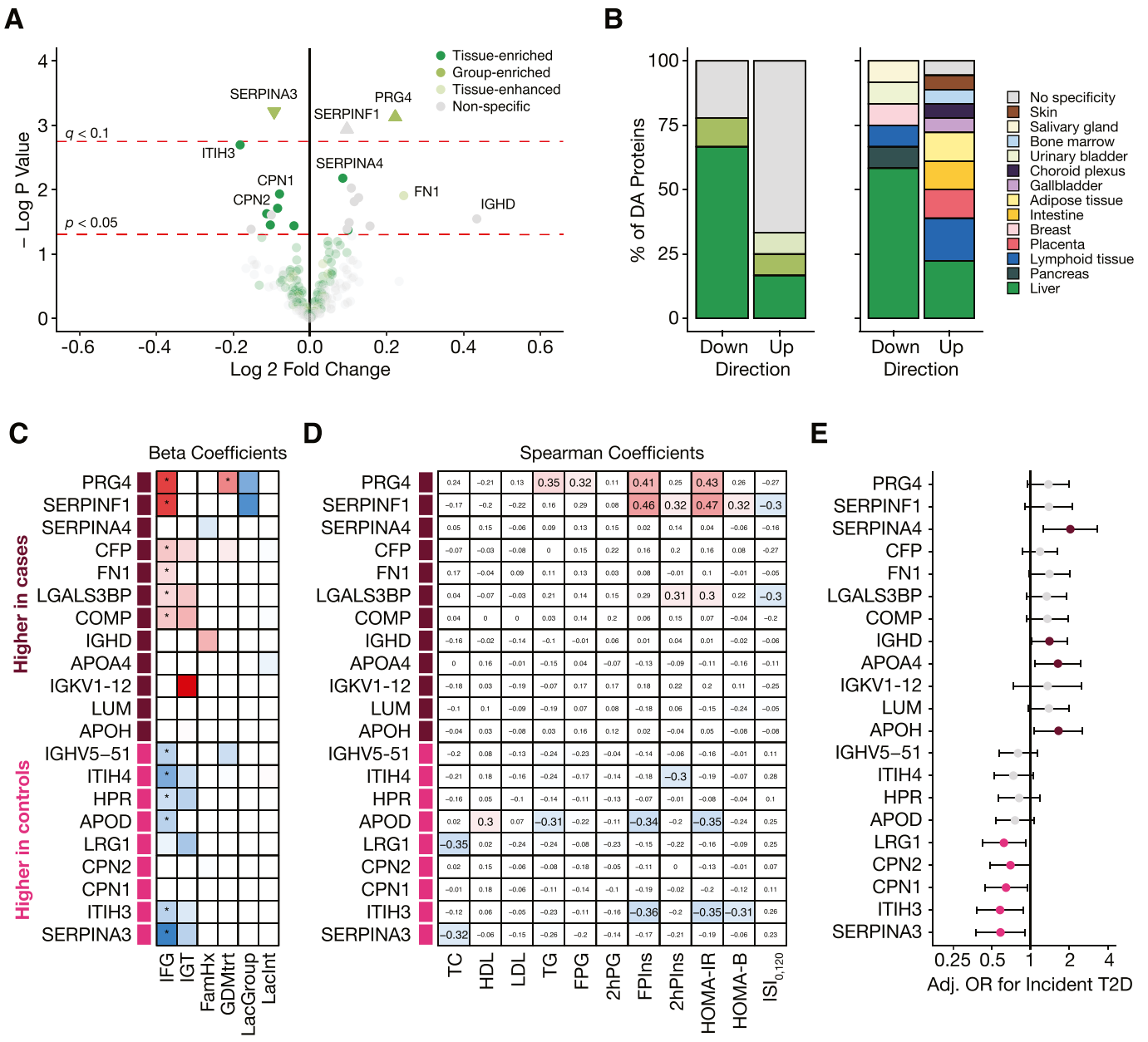

Fig. 2. Differential analysis of proteins associated with incident type 2 diabetes. Plasma protein profiling was performed on all participants in the current study. (A) Volcano plot showing fold changes (*x* axis) and *t*-test *p* values (*y* axis) of the 210 robustly quantified proteins (missing in >30 % of samples). (B) Distribution of liver specificity (left) and overall tissue enrichment (right) by fold change. (C) Cross-sectional associations between differentially abundant proteins and clinical risk factors for type 2 diabetes at baseline from logistic regression analyses. Asterisks indicate statistical significance after Benjamini-Hochberg (BH) correction for multiple comparisons ($q < 0.05$). (D) Spearman correlation coefficients between protein intensities and clinical characteristics, highlighting significant correlations (BH-adjusted $q < 0.05$ and $|r_s| > 0.3$). (E) Odds ratios per standard deviation increase in log2-transformed protein intensities for incident type 2 diabetes risk after adjusting for having impaired fasting glucose levels.

21 differentially abundant proteins ($p < 0.05$). Seven of the nine downregulated proteins (78 %) demonstrated some degree of tissue enrichment to the liver, whereas most upregulated proteins (75 %) mapped broadly to other origins such as the lymphoid tissues, intestines, adipose tissue, and placenta, (Fig. 2B), suggesting increased leakage or accumulation of tissue proteins into the circulatory system.

Given the clinical and biochemical characteristic differences between case and control groups at baseline, we next examined the impact of specific clinical biochemical characteristics on protein associations for future type 2 diabetes risk. Cross-sectional analyses revealed significant associations for having impaired fasting glucose levels at baseline with 12 of 21 differentially abundant proteins after multiple testing correction (BH adjustment, $q < 0.05$, Fig. 2C, Fig. S4). Interestingly, no differentially abundant protein was significantly associated with having impaired glucose tolerance at baseline, post-correction. Next, we identified statistically significant correlations (absolute $r_s > 0.3$, $q < 0.05$) with fasting plasma insulin levels and HOMA-IR scores (Fig. 2D) in four proteins: PRG4, PEDF, ITIH3, and apolipoprotein D. Since having impaired fasting glucose levels was widely associated with the differentially abundant proteins (Table S3), we included it in our multivariable conditional logistic regression analysis. At baseline, 9 of 21 differentially abundant proteins remained significantly associated with future type 2 diabetes risk (Fig. 2E), after adjusting for having impaired fasting glucose levels. In a stratified analysis, we demonstrated that the lists of proteins significantly associated with incident T2D within women who presented with impaired fasting glucose levels at baseline

(Fig. S5A) were completely distinct from those within women with normal fasting glucose levels (Fig. S5B); however, these proteins shared some overlap with those analyzed using the full cohort (Fig. SC–D). Overall, early differences in protein abundance may therefore reflect impairments in glucose regulation in the progression to type 2 diabetes.

We next assessed relationships for time to diabetes by stratifying the case group: women who developed T2D with 1 year postpartum (n = 35), 2 years postpartum (n = 38), and 4 years postpartum (n = 27). Case subgroups were compared against control subgroups with original pair-matching. Within the case group, time-to-diabetes was most strongly correlated with weight loss after pregnancy ($r_s = -0.28$, $p < 0.01$) and fiber intake ($r_s = -0.27$, $p < 0.01$) (Fig. S6A). As a result, these variables were included in the multivariable conditional logistic regression analyses (Fig. S6B). We identified three nearly exclusive sets of 14, 13, and 9 significantly associated proteins for a total of 34 unique proteins, of which eleven were differentially abundant between the full case and control groups (Fig. S6C). Our findings reveal unique insights into the timing of progression as a risk stratification tool.

3.4. Pathway analyses of differentially abundant proteins

To better understand the physiologic roles and regulatory mechanisms that may be disrupted in the progression from GDM to T2D, we performed a functional and pathway enrichment analysis using g:Profiler [29]. We uncovered 67 total terms and pathways represented by the 21 differentially abundant proteins (Table S4), save for IGHD. Half of these enriched terms and pathways could be assigned to four annotation clusters (Fig. 3): 1) inflammation, 2) protease inhibitor activity, 3) extracellular matrix components, and 4) lipid structure and function. Each cluster comprises at least 2 specific terms or pathways with at least 3 common proteins. Notably, the "inflammation" cluster captured 11 total proteins involved the acute-phase response, platelet function, and the complement cascade. We also identified an enriched protein complex in carboxypeptidase N involved in the complement cascade, wherein its two and only two subunits (CPN1 and CPN2) were both downregulated in the case group, compared to the control group. Furthermore, among the proteins involved in the "inflammation" cluster were four of five proteins (ITIH3, ITIH4, SERPINA3, SERPINA4) from the "protease inhibitor activity" cluster, suggesting a possible regulatory role in inflammation. The "extracellular matrix" cluster was unique in that it predominantly included upregulated proteins, as 9 of 12 proteins were increased at baseline. Finally, the "lipid structure and function" cluster highlighted an important cellular component in lipoproteins and interactions within the apolipoprotein L1 complex B. Non-clustered GO enrichments reflected more generalized GO terms and pathways, such as extracellular space, proteolysis, and protease regulation (Table S4).

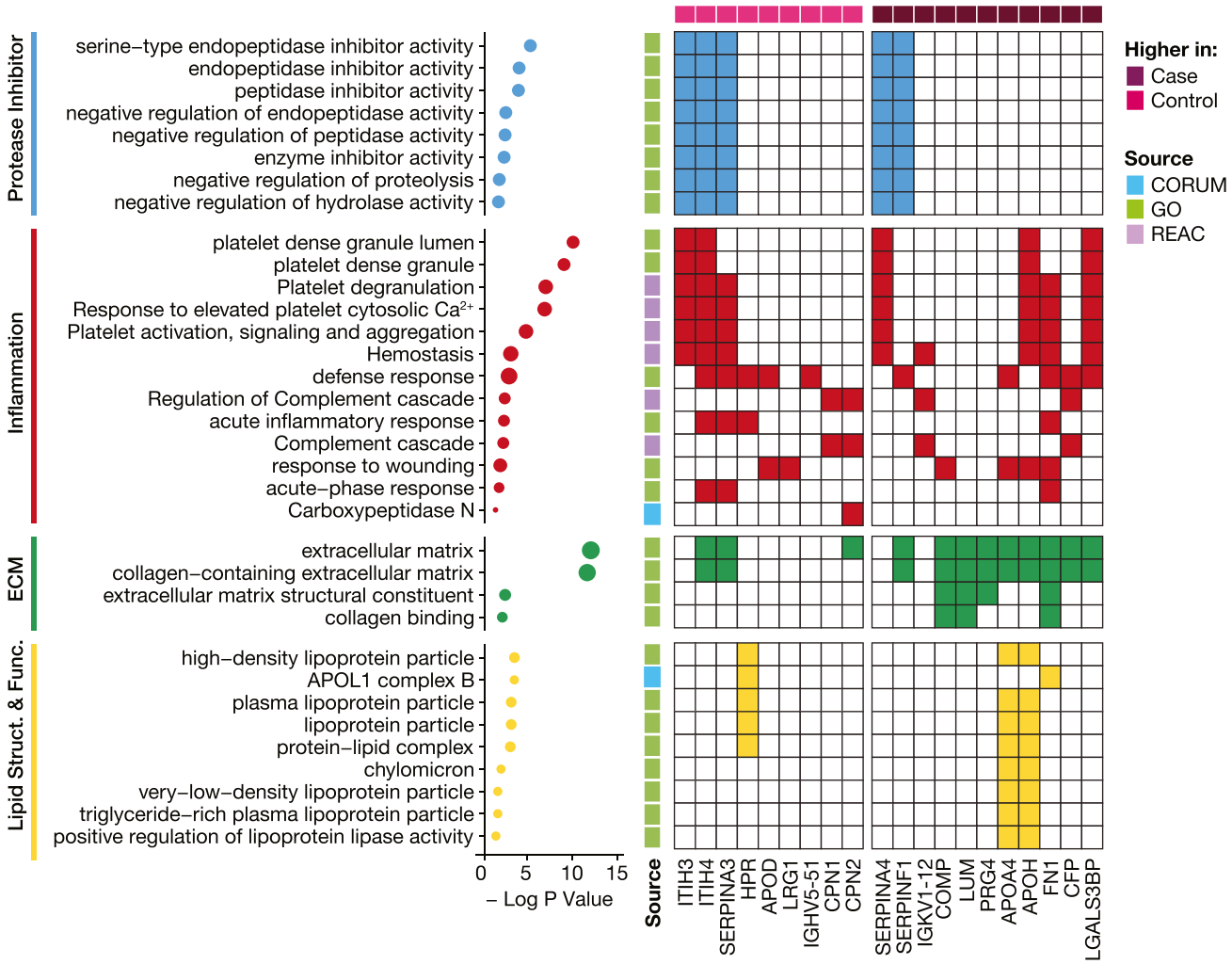

Fig. 3. Functional enrichment of differentially abundant proteins using g:Profiler. Half of the significantly enriched terms and pathways were organized into 4 main clusters: inflammation, protease inhibitor activity, extracellular matrix, and lipid structure and function. Non-clustered terms and pathways are not shown. The accompanying heatmap delineates the specific involvement of differentially abundant proteins. Proteins are also annotated and organized by fold change between the case (purple) and control (pink) groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Using this multi-resource approach, we also identified key associations between four proteins with two motifs in HNF1A and HNF1B, two genes that have been extensively linked in the development of type 2 diabetes [61,62].

From the previous stratified analysis, we identified unique lists of proteins associated with incident type 2 diabetes when separately examining women who presented with impaired fasting glucose levels and those with normal levels. These proteins also revealed distinct enrichments, where dysregulation of inflammation and the immune system may be more complicit in the progression to diabetes within women with impaired fasting glucose levels, and lipid dysregulation may be the focal point within those with normal fasting glucose levels (Fig. S5E–F).

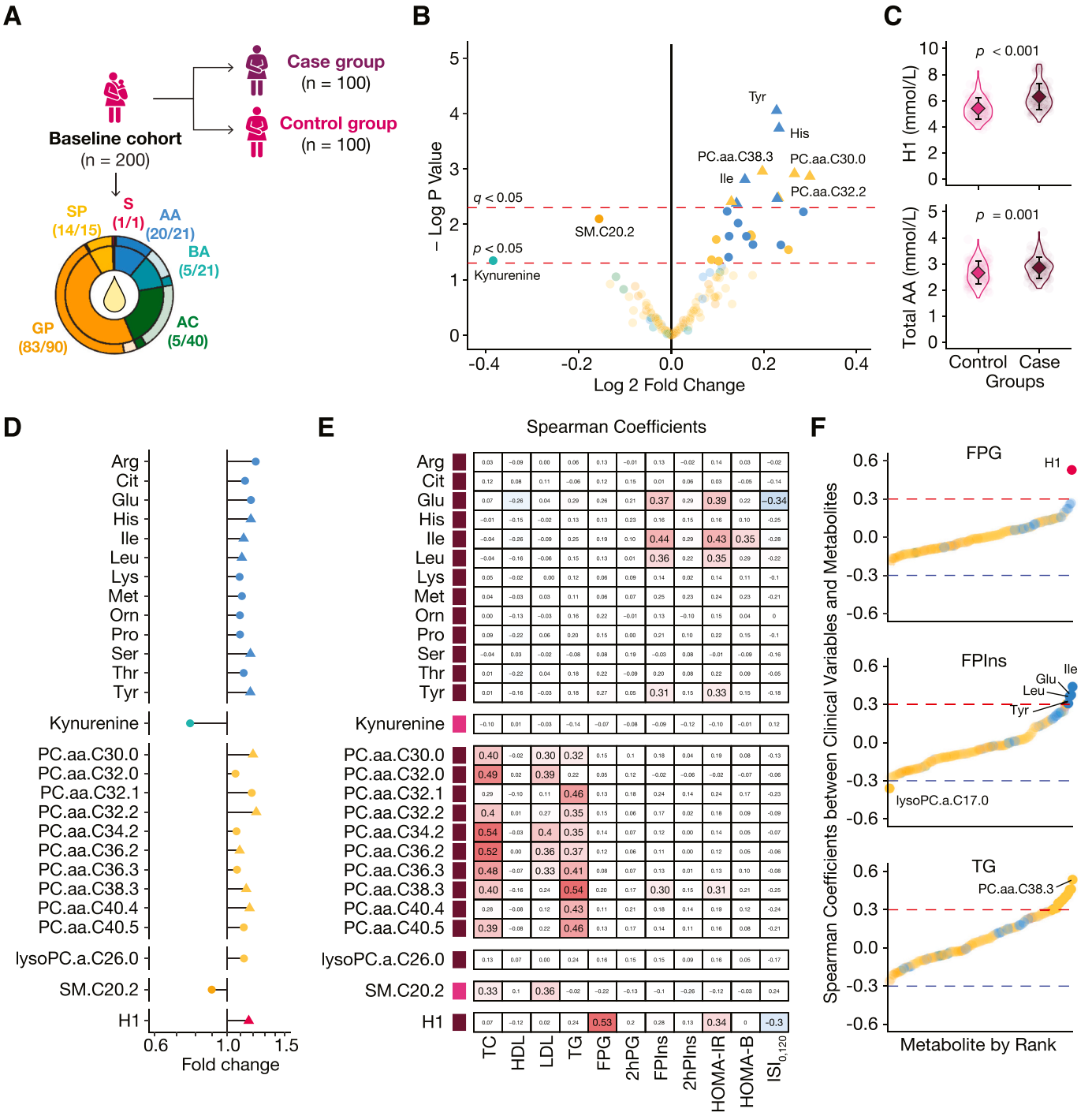

Fig. 4. Differential analysis of metabolites associated with incident type 2 diabetes. Plasma metabolomics was performed on all 200 participants in the current study. (A) Distribution of metabolites by subclass in the assay before (inner) and after (outer ring) removing metabolites that were missing from >30 % of samples. (B) Volcano plot showing fold changes (*x* axis) and t-test *p* values (*y* axis) of the 128 robustly quantified metabolites. (C) Summed concentrations of metabolite subclasses with significant differences between groups ($p < 0.05$). (D) Individual fold changes of differentially abundant metabolites. (E) Spearman's rank correlation coefficients r_s between metabolite concentrations and clinical characteristics, highlighting significant correlations (Benjamini-Hochberg-adjusted $q < 0.05$ and $|r_s| > 0.3$). (F) Spearman's rank correlation coefficients for fasting plasma glucose, insulin, and triglyceride levels. Amino acid, AA; biogenic amines, BA; acylcarnitines, AC; glycerophospholipid, GP; and sphingolipids, SL.

3.5. Metabolomic profile differences between groups

Given the metabolic disturbances associated with diabetes development, we next sought to integrate our proteomic analysis with metabolomic and lipidomic data for a comprehensive multi-omic investigation into the early underpinnings. Targeted mass spectrometry-based techniques using the Biocrates p180 assay were employed to accurately and reliably measure plasma concentrations of 128 robustly quantified metabolites belonging to 6 subclasses of analytes: amino acids, biogenic amines, acylcarnitines, glycerophospholipids, sphingolipids, and sugar (Fig. 4A).

We next characterized the baseline metabolite profiles between the case and control groups. At first glance, the metabolite profile featured an uneven distribution of analytes based on direction of change (Fig. 4B, Table S5), as 25 of 27 were significantly upregulated in the case group, compared to the control group ($p < 0.05$). Notably, hexose was the most statistically significant metabolite (fold change of 1.13, $p < 0.01$) (Fig. 4C). Differences at the subclass level were easily discerned, especially in total amino acid and glycerophospholipid levels (Fig. 4C, Table S6). Individually, 14 amino acids and 11 phospholipids were significantly increased, whereas the sphingolipid SM(C20:2) and biogenic amine kynurenine were downregulated (Fig. 4D). Five amino acids (His, Ile, Leu, Ser, and Tyr) as well as five acyl-alkyl-glycerophosphoslipids (C30:0, 32:2, 36:2, 38:3, and 40:4) remained significant after multiple testing correction (BH, $q < 0.05$), strengthening the utility of studying individual analytes. Correlations between metabolites and clinical biochemical characteristics revealed class-level patterns (absolute $r_s > 0.3$, $Q > 0.05$) (Fig. 4E), wherein amino acid levels (notably, Glu, Ile, Leu, and Tyr) displayed positive correlations with insulin-related measurements (e.g., fasting plasma insulin), and glycerophospholipids aligned with lipid-related measurements (e.g., triglyceride levels) (Fig. 4F). Besides hexose ($r_s = 0.53$, $q < 0.05$), fasting plasma glucose levels did not significantly correlate with any of the quantified metabolites (Fig. 4F).

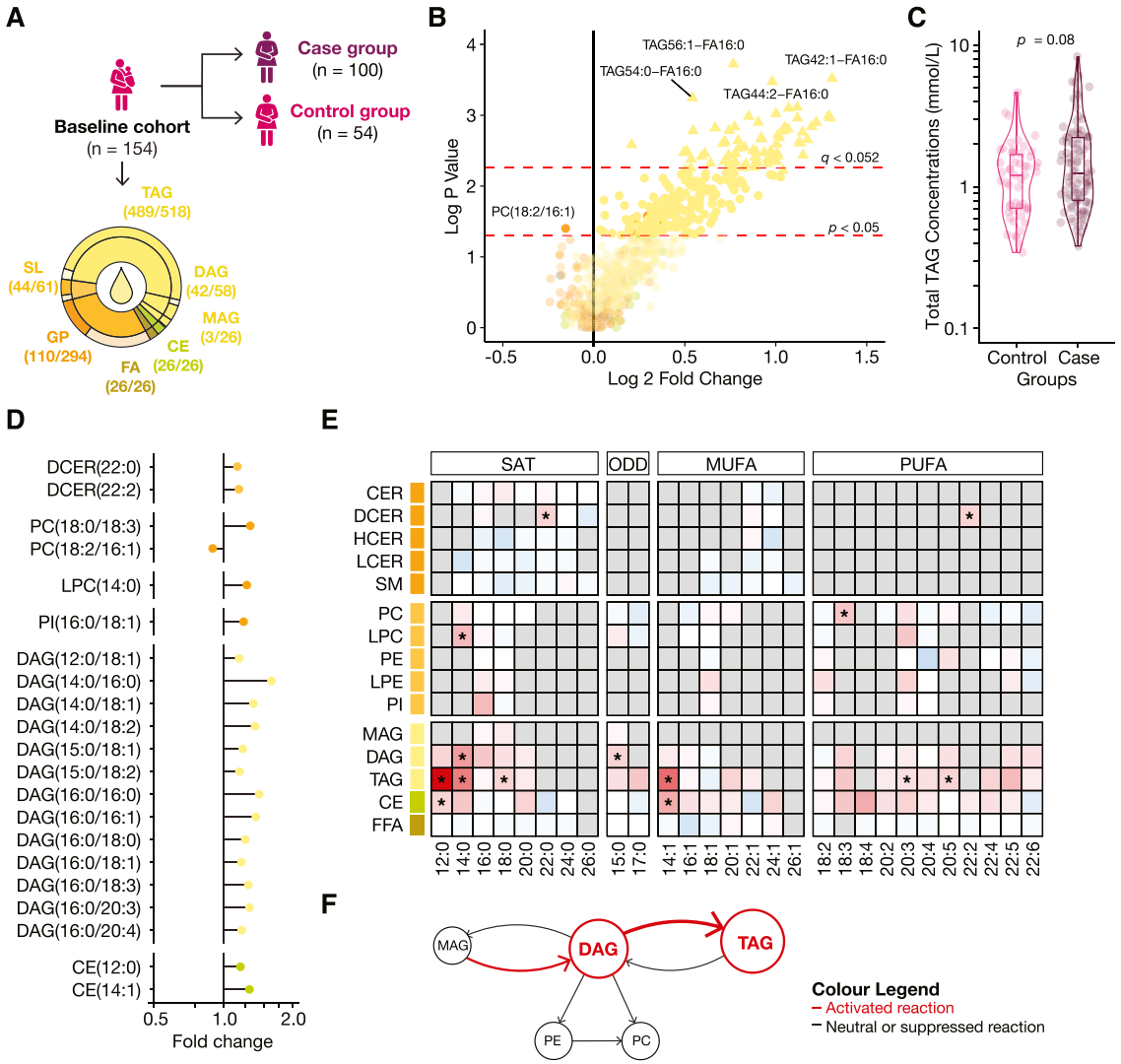

Fig. 5. Differential analysis of lipids associated with incident type 2 diabetes. Plasma lipidomics was performed on all 100 participants from the case group and 54 participants from the control group. (A) Distribution of lipid species by subclass in the assay before (inner) and after applying the filter cut-off of 100 % completeness (outer ring). (B) Volcano plot showing fold changes (*x* axis) and t-test *P* values (*y* axis) of the 739 robustly quantified lipids. (C) Summed concentrations of triglycerides by groups. (D) Individual fold changes of differentially abundant non-triglyceride lipids. (E) Fold changes of differentially abundant lipids reorganized by fatty acid type. (F) Pathway analysis using LIPID MAPS revealed an accumulation of TG via activated biosynthesis pathways. Nodes represent lipid subclasses, and directed edges (arrows) between nodes represent reactions. Edge thickness corresponds to the strength of a given direction based on z-scores of the pathways. Activated reactions are highlighted in red. Glycerophospholipid, GP; glycerolipids, GL, including monoglycerides, MG, diglycerides, DG, and triglycerides, TG; cholesterol esters, CE; fatty acids, FA; and sphingolipids, SL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Lipidomic profile differences between groups

Given the enrichment of lipoprotein cellular compartments within the protein profiles associated with future type 2 diabetes progression, we sought to more comprehensively investigate the lipid profiles using the Metabolon Complex Lipid Panel containing 1008 lipid species across 5 lipid subclasses (sphingolipids, glycerophospholipids, glycerolipids, cholesterol esters, and free fatty acids) (Fig. 5A). Unlike the other platforms, we applied a more stringent filter cut-off of 100 % completeness because lipidomic data was available for only 154 individuals in the current study. We therefore examined 739 total lipid species.

Remarkably, concentrations of 255 individual lipid species were significantly increased in the case group (Mann-Whitney test, $p < 0.05$), compared to the control group. No lipid species was significantly downregulated, which may reflect a hyperlipidemic state at baseline. As 234 (92 %) of these lipid species were triglycerides (Fig. 5B, Table S7), we also assessed differences at the subclass level. Summed concentrations of all measured triglyceride species were indeed elevated in the case group but did not achieve statistical significance ($p = 0.08$) (Fig. 5C, Table S8), likely owing to the reduced power and size of the control subgroup. The remaining upregulated lipid species were 13 diglycerides, 4 glycerophospholipids, 2 sphingolipids, and 2 cholesterol esters (Fig. 5D). Lipid species classified by fatty acid composition revealed significant increases in 6 triglyceride subclasses (12:0, 14:0, 18:0, 14:1, 20:3, 20:4), 3 sphingolipid subclasses (DCER22:0, DCER22:2), 2 diglyceride subtypes (14:0 and 15:0), 1 glycerophospholipid subclasses (LPC12:0, PC18:3) (Fig. 5E). Pathway analysis using LIPID MAPS [30,31] highlighted an activation of triglyceride biosynthesis pathways and suppression of glycerophospholipid pathways, culminating in the accumulation of triglycerides (Fig. 5F).

To investigate potential mediators for postpartum hypertriglyceridemia in women who later developed type 2 diabetes, we examined cross-sectional relationships between differentially abundant triglycerides and specific patient characteristics (Fig. S7). Triglycerides, as expected, significantly correlated with all fat-related biochemical clinical measures, except for LDL-cholesterol levels. We also demonstrated that elevated triglyceride levels were linked to increased insulin resistance, as shown by significant positive correlations for HOMA-IR scores (median $r_s = 0.45$; IQR 0.40–0.49, $q < 0.01$). Interestingly, triglyceride concentrations were not related to weight, waist circumference, and BMI ($p > 0.05$) and may better characterize adiposity than clinical anthropometry measures.

3.7. Multi-omic integration of proteins, metabolites, and lipids

By dissecting each profile individually, we have so far uncovered distinct insights into the future development of type 2 diabetes and now

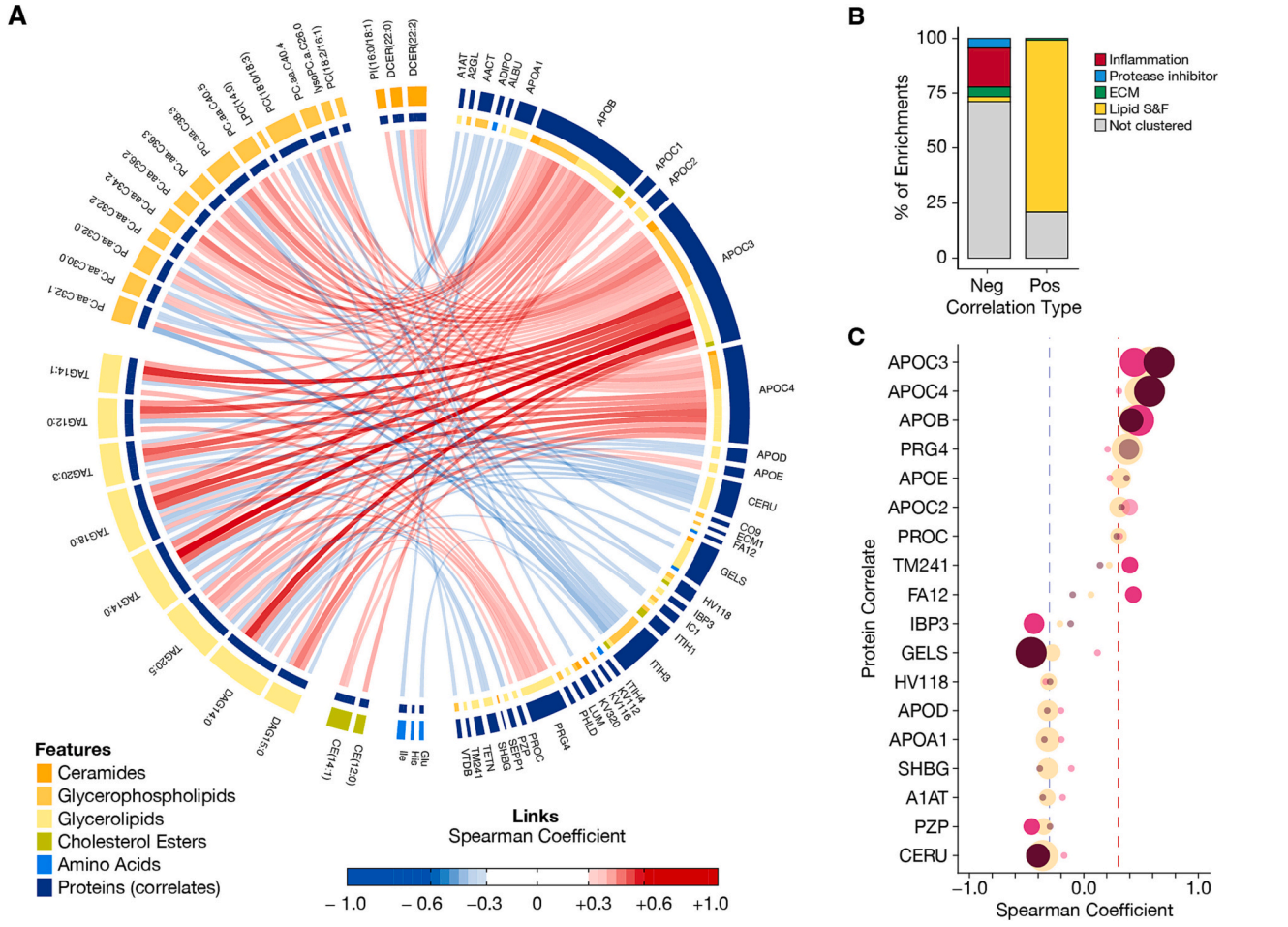

Fig. 6. Integrative omics. (A) Circos plot showing significant correlations of the full cohort among differentially abundant metabolites/lipids and protein correlates. Coloured links represent significant correlations (absolute Spearman's rank coefficient $r_s > 0.3$), with strength of the relationship depicted by opacity and direction by colour (blue, negative; red, positive). (B) Distribution of enriched terms and pathways associated with the negatively and positively correlated proteins by cluster. (C) Comparison of correlations within the case (purple) or control (pink) groups between differentially altered triglycerides and protein correlates. Full-cohort correlations are also shown (yellow). Size corresponds with the number of significantly correlated triglycerides ($|r_s| > 0.3$ for the full cohort; $|r_s| > 0.4$ for within-group subsets). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aim to better understand the interconnected nature, if any, of these distinct features. To assess possible interactions within common mechanisms, we first performed a joint pathway analysis on the differentially abundant proteins metabolites/lipids using MetaboAnalyst [27]. However, none of the significantly enriched pathways were simultaneously represented by both protein and metabolite/lipid classes (Table S9). While proteins of interest might not directly regulate lipid metabolism, they may carry out other essential functions, such transport and uptake (e.g., apolipoproteins). These multi-omic platforms therefore offer orthogonal information for a more complete understanding.

We next performed a correlation analysis to identify possible co-expression networks across features, namely identifying protein correlates of differentially altered metabolites and lipid subclasses. Of the 116 significant correlations ($|r_s| > 0.3$ and $q < 0.05$), the most common type of relationship was found between triglycerides and proteins. Proteins consistently correlated with individual analytes within the same class in the same direction (Fig. 6A), which further highlights a more generalized metabolic regulation. Of the 28 unique protein correlates, only six were differentially abundant at baseline between groups (Table S1) and may allude to the tighter regulation of proteins. Interestingly, 19 of 28 protein correlates were negatively correlated with metabolites and lipids. Notable exceptions include three apolipoproteins (B, C3, and C4), which were moderately-to-strongly and positively correlated to several lipid subclasses, shedding light on their critical roles in plasma lipid transport. Positive protein correlates (whose abundance changes in the same direction as concentrations of selected metabolites and lipids) were predominately related to lipid structure and function, accounting for roughly 80 % of pathways and terms, whereas negative protein correlates (whose abundance changed inversely) displayed a more diverse distribution that favoured the inflammatory response (Fig. 6B). Overall, these networks reveal a coexistence and possibly co-regulation of triglyceride levels and inflammation.

We next examined these cross-omic relationships within each group to determine early differences associated with progression (Fig. 6C). Correlations between apolipoprotein C (particularly C3 and C4) and differentially abundant glycerolipids were strengthened within the case group, compared to the control group (Table S10). Relationships between apolipoprotein B and glycerolipids however were strongest within the control group, suggesting a potential compensatory role. Within-group assessments also uncovered significant correlations that might have been missed when analysing the full cohort. Gelsolin, for example, was negatively correlated with triglycerides in the case group (r_s ranging from −0.49 to −0.40), but this relationship was neither replicated in the control group (ranging from 0.10 to 0.14) nor detected in the full cohort (r_s ranging from −0.29 to −0.24). Pregnancy-zone protein (PZP) and IGFB3 were most strongly and inversely related to triglyceride levels within the control group only, suggesting a loss in those who later developed diabetes. These group-specific relationships may reveal an early divergence toward future outcomes at baseline.

4. Discussion

In this nested case-control study, we examined the early underpinnings of progression to type 2 diabetes following a GDM pregnancy using integrated multi-omics. At 6–9 weeks postpartum (baseline), profile differences were detected across all platforms between women who developed type 2 diabetes within four years (case group) and those who did not (control group). Individually, these profiles offer distinct implications for wound healing and metabolic dysfunction in the future development of diabetes. Together, co-expression networks suggest that wound healing and metabolic dysfunction may contribute to progression via a positive feedback loop (Fig. 7).

We first demonstrated that specific metabolic adaptations of GDM – impaired fasting glucose levels, impaired glucose tolerance, and increased insulin resistance – are extant after delivery and more

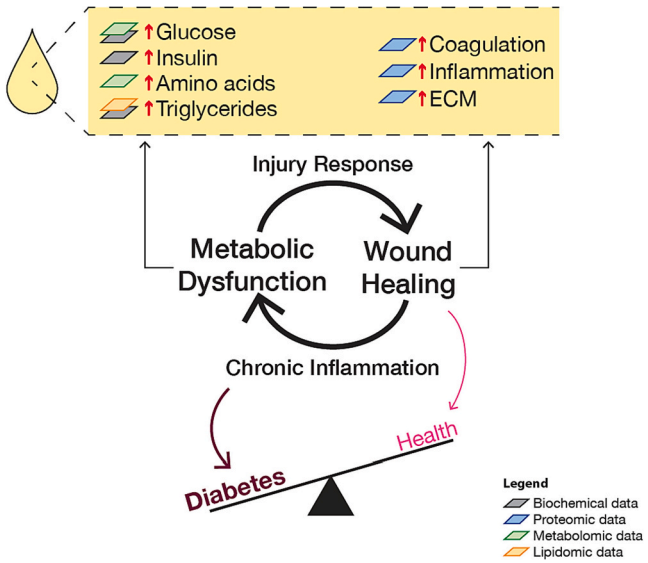

Fig. 7. Insights from early postpartum profiling into the progression from gestational diabetes (GDM) to type 2 diabetes. Circulatory profiles of women shortly after a GDM pregnancy uncovered perturbations in metabolic and wound healing processes that may contribute to the development of overt diabetes. Chronic dysmetabolism (marked by increased plasma levels of glucose, amino acids, and triglycerides) initiates and perpetuates the wound healing response, impairing the physiologic resolution of injury. Consequently, the accumulation of proteins involved in the activation of coagulation, inflammation, and extracellular matrix remodeling may exacerbate the chronic dysmetabolism and insulin resistance. This cycle ultimately accelerates the progression to diabetes in women with a GDM history.

prevalent in the case group. These adaptations were nuanced within the protein profiles, as differentially altered proteins for incident diabetes were also significantly associated with having impaired fasting glucose levels and, to a lesser extent, impaired glucose tolerance. Interestingly, we identified significant protein correlations for fasting plasma insulin levels, but not with fasting glucose levels, implicating involvement of these proteins in upstream causes of dysglycemia like insulin resistance. Protein profile differences highlighted key players involved in coagulation, inflammation, and extracellular matrix – three important phases of wound healing. Women who later developed diabetes generally had a higher abundance of proinflammatory proteins (e.g., PEDF, PRG4, FN1) and lower abundance of inflammatory regulators (e.g., SERPINA3, ITIH3, ITIH4, CPN complex, ITIH3), suggesting a chronic inflammatory state. While only PRG4 had been previously identified as a proteomic biomarker for gestational diabetes [32–34], our profiles share similar enrichments of complement and coagulation cascades with other studies on gestational diabetes or type 2 diabetes [32–36]. These proteomic findings therefore hint at an inflammatory state underlying this postpartum interface, likely in response to the lingering insulin resistance and dysglycemia at baseline.

Unlike the protein profile, metabolites and lipids were more susceptible to change at both individual and class levels. These profiles also broadened the scope of dysmetabolism beyond dysglycemia with global increases in amino acids and triglycerides, which further underscore a compromised resolution of GDM. Interestingly, decreased levels of plasma amino acids have been well-documented in normal pregnancy to support fetal development with a physiologic recalibration during the postpartum period [37,38]. Evidently, GDM distorts the pregnancy profile with significant dysregulation of individual plasma amino acids [39,40]. A comparison between pregnancy and postpartum levels indicated that higher postpartum levels of branched-chain amino acids may be distinctive of GDM [41]. In the early postpartum period, we previously demonstrated that increased amino acid levels were often

sustained or exacerbated during the two-year follow-up after a GDM pregnancy in women who later developed diabetes [18]. Similar to our findings, specific amino acids have been previously linked to increased insulin resistance [42–44] and may therefore signal, if not contribute to, future development of diabetes. Longitudinal analyses of pregnancy profiles highlight a physiologic accumulation of triglyceride in the maternal system, with an inflated response in GDM pregnancy [38,45,46]. Additionally, given the comorbidity of obesity and diabetes, it was not surprising that elevated triglyceride levels have been previously linked to progression [20,47–49]. While plasma triglycerides are believed to rapidly decrease following delivery, we speculate that those who develop type 2 diabetes are more likely to sustain a subclinical hypertriglyceridemia or have a slower decline in plasma triglyceride levels.

Our multiomic approach uncovered two contemporary hallmarks of diabetes: inflammation (through proteomics) and dysmetabolism (through metabolomics and lipidomics). These hallmarks are indeed interconnected, as evidenced by our co-expression networks of differentially abundant triglycerides and proteins involved in the complement cascade. An emerging hypothesis of diabetes pathophysiology suggests that imbalances in metabolites and nutrients activate the wound healing response [50–52]. Normal wound healing delivers a complete return to normal structure and function [53] – a prime example is the postpartum recovery following a healthy pregnancy. Repeated or prolonged exposure to injury however can culminate in a hypercoagulable state, unresolved inflammation, and scar tissue formation [53]; these hallmarks of a perturbed wound healing state have been extensively described in diabetes [52,54,55]. Our findings provide more evidence that this positive feedback loop between metabolic dysfunction and inflammation, termed meta-inflammation, may persist at 6–9 weeks postpartum, albeit in a milder form compared to GDM pregnancy, and contribute to the re-emergence of diabetes in later life.

The current study has many strengths. We are first to apply multidimensional profiling techniques in combination with extensive clinical characterization on a diverse cohort of women with GDM who later developed type 2 diabetes, leveraging an extensively characterized SWIFT population of 1035 women after a GDM pregnancy (with 95 % retention from recruitment to follow-up and 98 % testing rates captured by electronic medical records). Furthermore, our systematic gold standard testing procedures, including multi-timepoint OGTT measurements of plasma glucose and insulin, ensure accurate classification of glycemic status during and after pregnancy. Another major strength is the racial and ethnic diversity of our study participants (87 % are Asian, Black, or Hispanic women), which befits the population of women with GDM in the United States, especially those at increased risk of both GDM and type 2 diabetes. Importantly, our baseline profiles provide a wide-range window into the postpartum period, a largely unexplored timepoint between GDM to type 2 diabetes that offers early insights into progression. We revealed key differences in each of the plasma profiles, whereby protein profiles featured a more balanced distribution of upregulated and downregulated changes and metabolite/lipid profiles depicted both individual- and class-level changes. These lopsided profiles also reflected the nature of proteins, metabolites, and lipids in physiology, supporting our application and integration of orthogonal approaches.

Limitations of this study will serve as future directions for our investigation. Notably, with rapid breakthroughs in artificial intelligence, our work can be bolstered by machine learning to expand the proteome coverage, to build prediction models, and to assess prognostic performance [56–58]. In this study, we decided against current library matching algorithms to maximize reproducibility and specificity. Although other proteomic analyses have quantified larger numbers of proteins, differential expression was often detected at similar concentration ranges as found in our study [25,59,60]. Our workflow therefore provides a suitable and intact examination of plasma profiles. As for biomarker discovery and prediction modeling, our study lacked a suitable independent validation cohort, namely of women with glucose tolerance testing during the early postpartum period after a GDM pregnancy. This nested case-control study design also restricts the investigation of time-to-diabetes as an important outcome, and as a result, differences in women who progress from GDM to diabetes at other time points after 4 years were not captured by our current analysis but may be assessed in the future within the entire SWIFT cohort (which is in ongoing follow-up). Additionally, our matching strategy does not comprehensively account for all possible confounders and may reduce generalizability of the study. The singlicate analysis is another limitation, influencing variability; though, our analysis of only fasting plasma samples in part alleviates this concern (along with our quality control assessment on ten technical replicates). Overall, our study provides interesting avenues for future work with broader and larger cohorts to better assess and deconstruct the heterogeneity of the progression from GDM to T2D over time.

5. Conclusion

The postpartum period, especially after a GDM pregnancy, encapsulates a critical crossroad for the return to the pre-pregnancy state or progression to type 2 diabetes. We uncovered an inflammatory and dysmetabolic state, which was more pronounced and interconnected in women who later developed diabetes. Multidimensional approaches, such as integrative omics combined with machine learning, may provide a full-scope dissection into the precise mechanisms underlying this heterogeneous disease.

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CRediT authorship contribution statement

Julie A.D. Van: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Yihan Luo:** Writing – review & editing. **Jayne S. Danska:** Funding acquisition, Writing – review & editing. **Feihan Dai:** Writing – review & editing. **Stacey E. Alexeeff:** Writing – review & editing. **Erica P. Gunderson:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Hannes Rost:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Michael B. Wheeler:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

All relevant proteomic data have been deposited onto the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041243 (https://www.ebi.ac.uk/pride/login; reviewer_pxd041243@ebi.ac.uk). Previously published metabolomic and lipidomic data can be accessed through the Harvard Dataverse (https://dataverse.harvard.edu/dataverse) under respective identifiers, KUDDSF and HQ55W6.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2023.155695.

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