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Original research

# Gestational diabetes is driven by microbiota-induced inflammation months before diagnosis

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**ABSTRACT**

**Objective** Gestational diabetes mellitus (GDM) is a condition in which women without diabetes are diagnosed with glucose intolerance during pregnancy, typically in the second or third trimester. Early diagnosis, along with a better understanding of its pathophysiology during the first trimester of pregnancy, may be effective in reducing incidence and associated short-term and long-term morbidities.

**Design** We comprehensively profiled the gut microbiome, metabolome, inflammatory cytokines, nutrition and clinical records of 394 women during the first trimester of pregnancy, before GDM diagnosis. We then built a model that can predict GDM onset weeks before it is typically diagnosed. Further, we demonstrated the role of the microbiome in disease using faecal microbiota transplant (FMT) of first trimester samples from pregnant women across three unique cohorts.

**Results** We found elevated levels of proinflammatory cytokines in women who later developed GDM, decreased faecal short-chain fatty acids and altered microbiome. We next confirmed that differences in GDM-associated microbial composition during the first trimester drove inflammation and insulin resistance more than 10 weeks prior to GDM diagnosis using FMT experiments. Following these observations, we used a machine learning approach to predict GDM based on first trimester clinical, microbial and inflammatory markers with high accuracy.

**Conclusion** GDM onset can be identified in the first trimester of pregnancy, earlier than currently accepted. Furthermore, the gut microbiome appears to play a role in inflammation-induced GDM pathogenesis, with interleukin-6 as a potential contributor to pathogenesis. Potential GDM markers, including microbiota, can serve as targets for early diagnostics and therapeutic intervention leading to prevention.

**INTRODUCTION**

Gestational diabetes mellitus (GDM), or development of glucose intolerance during pregnancy

**WHAT IS ALREADY KNOWN ON THIS TOPIC**

- ⇒ The incidence of gestational diabetes mellitus (GDM) is increasing worldwide.
- ⇒ Early prediction of GDM may reduce short-term and long-term complications to the mother and the offspring.
- ⇒ At later stages of pregnancy, the gut microbiome of women diagnosed with GDM is different from the microbiome of women without GDM.
- ⇒ Insulin resistance has been associated with elevated secretion of proinflammatory cytokines.

**WHAT THIS STUDY ADDS**

- ⇒ Gut microbiome, metabolome and inflammatory markers were profiled during the first trimester of pregnancy in 394 women.
- ⇒ Significant differences were found in these markers between women who would and would not later develop GDM.
- ⇒ The GDM phenotype was transferred to germ-free mice following faecal microbiota transplant from women in their first trimester of pregnancy.
- ⇒ Accurate prediction of GDM development was made based on first trimester biomarker profiles and clinical data.
- ⇒ This study suggests diagnosis of GDM/GDM risk can be made earlier allowing for earlier management or even complete prevention.

in women without diabetes, occurs when the pancreas cannot produce enough insulin to balance insulin-inhibiting effects of placental hormones (viz. oestrogen, cortisol and human placental lactogen).<sup>1</sup> Approximately 10% of pregnant women worldwide are diagnosed with GDM. Risk factors include non-white ethnicity, increased maternal age, obesity, family history of diabetes and history of giving birth to large

**HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY**

- ⇒ Recognition of women at risk of GDM at an early stage of pregnancy, with appropriate risk stratification, may allow specific recommendations for prevention of the disease—currently by lifestyle modification and in the future perhaps by specific pre/pro/postbiotic supplementation.
- ⇒ If GDM can be prevented, there would be a major reduction in adverse outcomes of GDM, for the mother and offspring, in both the short term and long term.

infants. Consequences of GDM include a wide range of obstetrical and metabolic complications for both the mother (eg, pre-eclampsia, type 2 diabetes and cardiovascular diseases) and the neonate (mainly macrosomia and hypoglycaemia).<sup>2</sup> Many complications are preventable if GDM is detected and appropriately managed and good glycaemic control is achieved by nutrition, exercise and insulin administration, if necessary, along with heightened monitoring during labour and delivery,<sup>3</sup> but earlier detection might allow for complete amelioration of GDM-associated short-term and long-term risks.

The incidence of GDM is increasing worldwide, due primarily to the increase in prevalence of overweight and obesity, advanced maternal age and growth of at-risk populations.<sup>4–6</sup> As such, it is important to expand early-prediction efforts towards reducing its negative consequences. To date, few studies have examined biomarkers of GDM in the first trimester (T1).<sup>7,8</sup> Additionally, while gut microbial dysbiosis has been associated with diabetes,<sup>9</sup> and a recent study has associated gut dysbiosis with GDM in the third trimester (T3),<sup>10</sup> few have focused on T1.<sup>8,11–15</sup>

We sought to identify biomarkers of GDM in T1 of pregnancy. First, we comprehensively profiled the T1 gut microbiome, metabolome and inflammatory cytokine profiles of women who would and would not later be diagnosed with GDM. We then investigated whether the early pregnancy microbiome drove GDM development using germ-free (GF) mice. Finally, we used a machine learning approach to predict GDM based on patient characteristics, T1 microbiome and clinical information, to identify earlier time frames for therapeutic intervention.

**METHODS****Pregnant women****Primary prospective cohort**

We enrolled a prospective cohort followed throughout pregnancy (online supplemental figure 1). Upon screening for GDM in the second trimester (T2; screening method described in online supplemental methods), women were retroactively classified as ‘would go on to develop GDM’ and ‘would not go on to develop GDM’. This main prospective cohort included 394 pregnant women aged 18–40 years recruited between gestational ages (weeks+days) 11+0–13+6 at women’s health centres of Clalit HMO (Dan Petach Tikva District, Israel) during the years 2016–2017. Exclusion criteria included: type 1 or type 2 diabetes mellitus diagnosed before pregnancy (all other chronic diseases were documented in the database); *in vitro* fertilisation or hormonal therapy in the previous 3 months; use of antibiotics in the previous 3 months and multiple gestation. Initially, 400 women were recruited, but 4 did not provide any samples and 2 did not meet study criteria upon further examination

of medical records (one with antibiotics use, one with type 2 diabetes; online supplemental figure 1). Thus, 394 women were followed through 27–31 weeks of pregnancy; in this study, no women were lost to follow-up as following initial recruitment, all other data (namely GDM diagnosis) could be obtained from digital medical records. Weight and height were assessed at the time of recruitment and blood and faecal samples collected (see online supplemental methods). Dietary consumption (24-hour recall), physical activity (24-hour recall), sleeping hours (3-day recall), stress (validated questionnaire<sup>16</sup>), employment and education details (at recruitment) were recorded. Other maternal demographics, clinical and obstetrical data including pregnancy follow-up and comorbidities were extracted from medical records.

**Secondary cohort**

Since GDM incidence in Israel is about 10%,<sup>17</sup> a secondary cohort of pregnant women was also recruited. Patients with GDM were enrolled in a cohort study at 24–28 gestational weeks at Rabin Medical Center between the years 2016 and 2017. Exclusion criteria for this cohort were the same as for the main cohort. Medical chart review was performed to identify all demographic and clinical characteristics from T1. Clinical data, but not biological samples, from this secondary cohort are included in the study.

**Additional cohorts**

In addition to the above cohorts, for faecal microbiome transplant (FMT) experiments in GF models, two additional independent cohorts were included (see online supplemental methods).

**Biomarker analysis in the primary cohort**

Fasting glucose, liver enzymes and HbA1c were extracted from medical records and serum cytokine and hormone panels performed (online supplemental methods). Bacterial DNA was extracted, amplified (V4 region of the 16S rRNA gene) and sequenced (Illumina MiSeq) from all faecal samples as described in the online supplemental methods. QIIME2 V.2019.4<sup>18</sup> was used for read pre-processing (pipeline in online supplemental methods). Faecal short-chain fatty acid (SCFA) extraction and untargeted metabolomics methods are also described in the online supplemental methods.

**FMT into GF mice**

Transplantation experiments were performed using faecal samples from the primary prospective cohort and the two additional cohorts (see online supplemental methods).

**Prediction**

To predict GDM, we developed a prediction model using our prospective cohort (identified T1 biological markers and clinical data) as well as clinical data from our secondary cohort. We checked each combination of the following components: (1) cytokines, (2) microbiome, (3) general clinical information and (4) food questionnaires. The accuracy of the prediction was assessed using the area under the curve of the test set, in a 20%/80% test/training set division and a fivefold cross-validation (see online supplemental methods). To examine generalisability of our model, we applied the classifier to an independently published dataset from a Chinese cohort of 98 pairs of pregnant women with and without GDM (matched) who provided a faecal sample in week 10–15 of pregnancy.<sup>8</sup> We trained the

model on our primary cohort and tested the model performance on the Chinese cohort.

### Statistical analysis

Full statistical methods are presented in the online supplemental methods. Briefly, unless otherwise specified, statistical analysis was done using non-parametric Mann-Whitney U tests followed by false discovery rate (FDR) correction. Mantel's correlations between study features were performed. Association of microbial features with GDM was done by Spearman's rank correlations compared with a background distribution followed by a linear model to control for main risk factors. For untargeted metabolomics, the differential abundance of the metabolites between the groups was identified by Student's t-tests and FDR correction. Microbial features of FMT-recipient mice were associated with GDM using MaAsLin2.<sup>19</sup> The MetaCyc pathway abundance in mouse faeces was predicted using PICRUSt2.<sup>20</sup>

### Data availability

All sequencing data were submitted to European Bioinformatics Institute (EBI) (project accession number ERP143097). Metabolomics data were deposited at 10.5281/zenodo.6581068.<sup>21</sup> Ethics statement and patient and public involvement are described in online supplemental methods.

## RESULTS

### Study design

We prospectively recruited 394 women during T1, 44 (11%) of which went on to develop GDM, as diagnosed by glucose tolerance test (GTT) during the second trimester of pregnancy. The other 350 women served as the control group, hereafter 'healthy pregnant women' (online supplemental figure 1). Of the recruited women (regardless of GDM status), 8 suffered spontaneous abortion, 7 delivered preterm and 11 had gestational hypertension or pre-eclampsia. In addition, 4 had polycystic ovary syndrome and 25 had hypothyroidism. These were not exclusion criteria.

Of the 34 women in the GDM group who had blood work on file before pregnancy, 2 had high HbA1c; none had high glucose. Women diagnosed with GDM exhibited other common risk factors (table 1) such as higher maternal age and pre-pregnancy body mass index (BMI). Following pregnancy (6 weeks–6 months), we also examined HbA1c (or glucose) levels of these

women and found one woman with high HbA1c level (out of six who did this blood work) and none with impaired glucose levels (fasting test/75 g oral GTT, out of 22). While beyond the timeline of this T1 study, among women later diagnosed with GDM, dietary consultation/lifestyle change was not sufficient for nine women who therefore received medication to control their GDM.

When examining explained variance between parameters measured (microbiome, SCFA, metabolome, cytokines, hormones, diet and lifestyle; figure 1A), using a Mantel test, we found that the T1 gut microbiome significantly explained the variance of most measurements and was most tightly correlated with the faecal metabolomic profile (figure 1B).

### Women with GDM exhibit elevated levels of serum inflammatory cytokines and low levels of SCFAs in T1

Following evidence of elevated inflammatory biomarkers in women diagnosed with GDM,<sup>22</sup> we profiled 10 plasma cytokines, chemokines and hormones in both the GDM (n=35) and control (n=78) groups and found elevated levels of proinflammatory cytokines (interleukin (IL)-4, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor and tumour necrosis factor- $\alpha$ ) among the GDM group (figure 1C;  $p < 0.05$ , FDR-corrected Mann-Whitney U tests) but no differences in leptin and insulin. This result was robust when controlling for BMI and age (see the online supplemental methods;  $p < 0.05$ , linear model with age and BMI as fixed or random effects).

Another possible early biomarker for GDM are SCFAs, which promote glucose homeostasis and suppress inflammatory response. We found a significant reduction of two branched SCFAs (BSCFAs), isovalerate and isobutyrate, in the GDM group (figure 1D;  $p < 0.05$ , FDR-corrected Mann-Whitney U tests) and a similar trend for valerate ( $p = 0.09$ ).

### Gut microbiome is associated with GDM pathogenesis

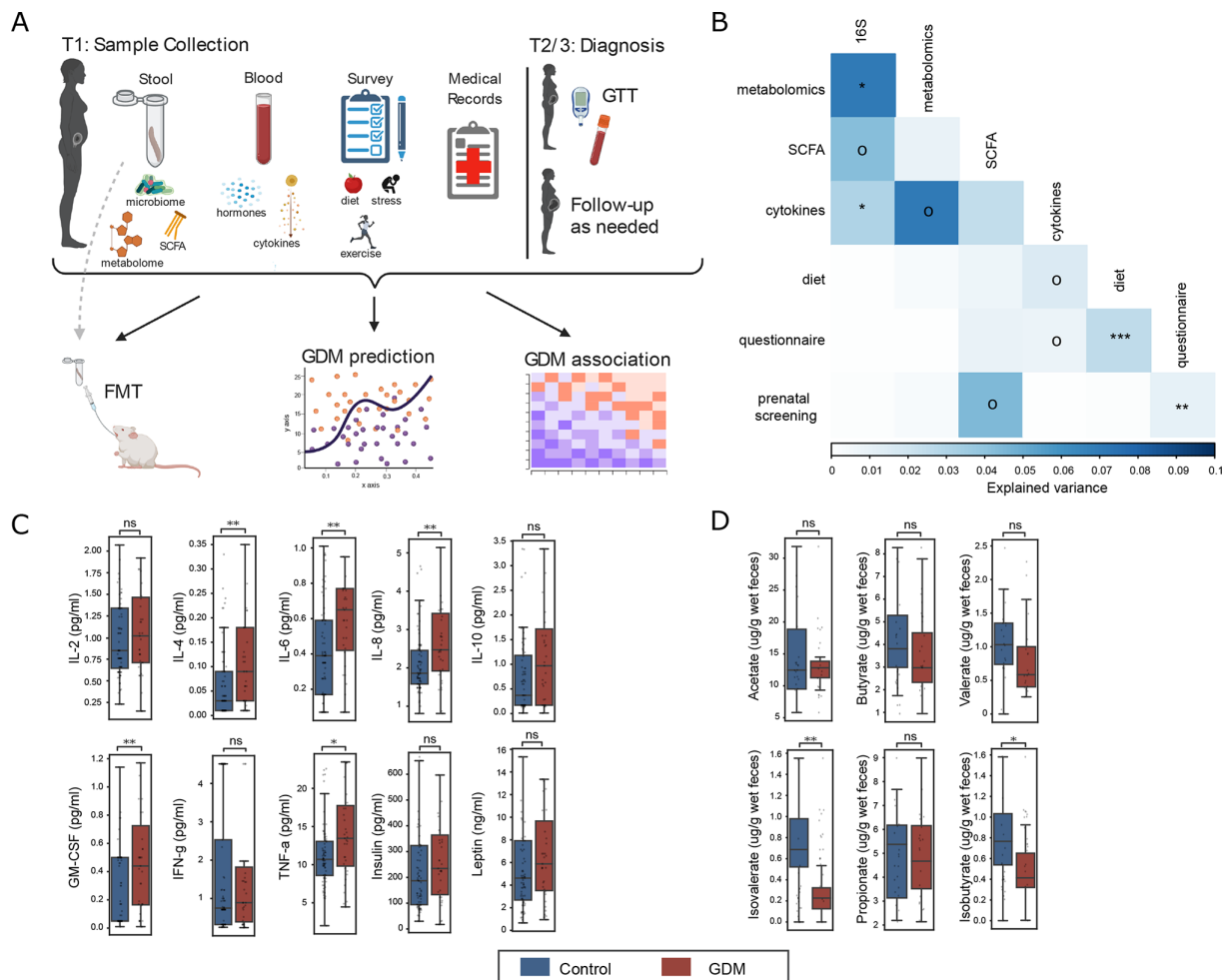
A number of studies have suggested that the gut microbiome is altered in women with GDM, post-GDM diagnosis. In our study, we did not find differences in T1 gut microbiome  $\alpha$ -diversity between women who would and would not develop GDM. Principal coordinate analysis of unweighted UniFrac distances demonstrated that the microbial communities of healthy women and women with GDM trend toward significant differences (figure 2A;  $p = 0.06$ , permutational multivariate analysis of variance;  $p = 0.23, 0.05, 0.38$  for Bray-Curtis, Jaccard and weighted UniFrac, respectively), supported by results of differential abundance analyses (below). Notably, when fitting a linear model to the distance matrix with GDM outcome and the risk factors age and BMI, widely associated with GDM (sequentially using adonis2, see the online supplemental methods), none of the variables were significant.

We next aimed to characterise the specific subset of differentially abundant bacteria: 1 bacterial species was over-represented and 16 bacteria under-represented in the GDM group. When repeating this analysis while controlling for age and BMI, we found 15 under-represented species in the GDM group (figure 2B), only 6 of which intersected with the prior, uncontrolled analysis. Controlling for confounding variables allowed us to distinguish between microbial species associated with main risk factors of GDM and the disease itself. We found a lower abundance of *Prevotella* in T1 samples of women who would develop GDM, and this result was replicated in mice (below).

**Table 1** Cohort description

	Control	GDM	P value
Women	350	44	
BMI	22.6 $\pm$ 4.0 (325)	28.2 $\pm$ 7.7 (43)	<0.0001
Age (years)	31.2 $\pm$ 4.43 (350)	33.1 $\pm$ 3.77 (44)	<0.01
Gestation	2.38 $\pm$ 1.44 (349)	2.93 $\pm$ 1.8 (44)	0.02
Parity	0.97 $\pm$ 1.04 (349)	1.2 $\pm$ 1.0 (44)	0.16
Fasting glucose (mg/dL)	82.46 $\pm$ 7.08 (327)	89.30 $\pm$ 14.1 (42)	<0.0001
Delivery week	39.1 $\pm$ 1.73(297)	38.7 $\pm$ 1.2 (39)	0.14
Newborn weight (kg)	3.2 $\pm$ 0.46 (308)	3.24 $\pm$ 1.2 (40)	0.66
Stress (rank: 1–4)	1.41 $\pm$ 0.59 (232)	1.57 $\pm$ 0.63 (30)	0.17
Education (years)	15.36 $\pm$ 2.3 (251)	14.75 $\pm$ 2.17 (33)	0.15
Current smoker	32	11	<0.01*
Non-smoker	280	28	
Prior smoker	13	1	

Data are presented as mean $\pm$ SD. P values based on Student's t-test. Parentheses represent sample size.  
\*P value based on Fisher's exact test.  
BMI, body mass index; GDM, gestational diabetes mellitus.



**Figure 1** First trimester blood and faecal biomarkers in women later diagnosed with GDM. (A) Sampling strategy and study design. Samples were collected in first trimester (T1). Stool was collected to profile gut microbiome (GDM:  $n=28$ , control:  $n=236$ ), metabolome ( $n=15$  age/BMI-matched pairs) and SCFAs ( $n=20$  age-matched pairs) and to validate results when transplanted into germ-free mice. Blood samples were used to profile cytokines and hormones (GDM:  $n=35$ , control:  $n=78$ ). Lifestyle surveys and medical records were collected from all participants. (B) Variance explained (square of the Mantel statistic) between all pairs of data types (Mantel test). (C) Serum levels of cytokines and hormones for GDM and control women (false discovery rate (FDR)-corrected Mann-Whitney U tests). (D) Concentration of faecal short-chain fatty acids (FDR-corrected Mann-Whitney U tests). Boxplots indicate the median and IQR; whiskers show IQR $\times 1.5$ .  $^{\circ}P<0.1$ ,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ . BMI, body mass index; FMT, faecal microbiota transplant; GDM, gestational diabetes mellitus; GM-CSF, granulocyte-macrophage colony-stimulating factor; GTT, glucose tolerance test; IFN, interferon; IL, interleukin; ns, not significant; SCFA, short-chain fatty acid; TNF, tumour necrosis factor.

#### Glucose impairment and elevated IL-6 levels of women with GDM were phenocopied to mice by FMT

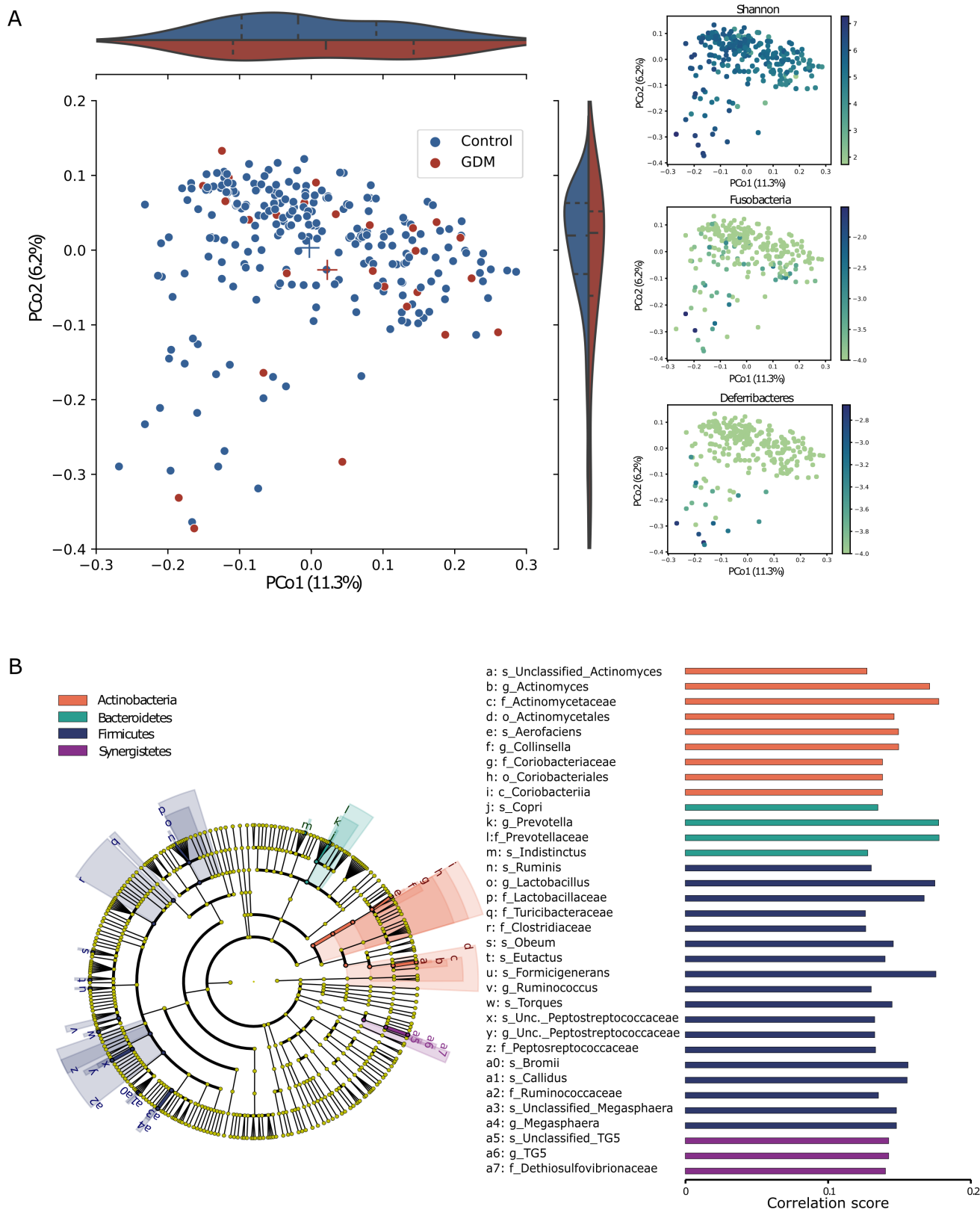
To examine the causal role of the gut microbiome in the pathogenesis of GDM, faecal samples of age-matched and BMI-matched GDM and control samples from the primary cohort were transplanted to GF female mice (figure 3A). Microbiota characterisation was performed 7 and 21 days post-FMT. The GF mice acquired an average of 42 and 48 taxa from donor samples 7 and 21 days post-transfer, respectively. The recipient mice shared  $\sim 60\%$  of their taxa with the donor on day 7 and  $\sim 55\%$  on day 21 (online supplemental figure 2). On day 7, the microbial communities were significantly different between GDM-recipient and non-GDM recipient mice (figure 3B). Consistent with our observation in women, *P. copri* was found to be reduced in GDM-recipient mice (figure 3C). GTTs revealed GDM-recipient mice exhibited impaired glucose tolerance (figure 3D).

Further, the GDM-recipient mice exhibited elevated levels of both IL-6 (in agreement with our findings in women with GDM)

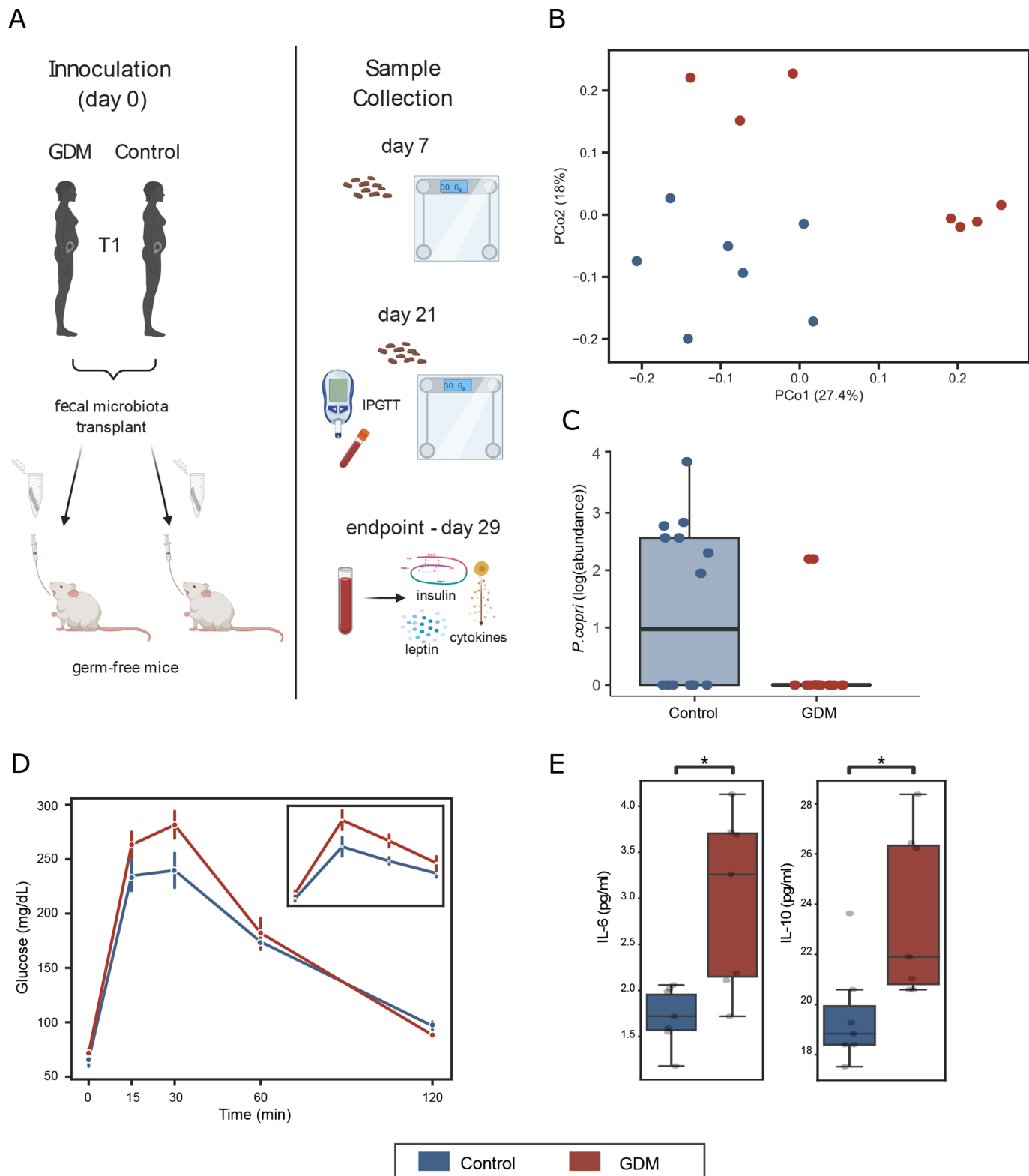
and IL-10 relative to the control-recipient mice (figure 3E and online supplemental table 1). No differences were found for insulin or leptin levels (online supplemental table 2). We found further support for the role of gut microbiota in GDM pathogenesis with FMT from two additional GDM cohorts (Finnish and American women) (figure 3D; online supplemental figure 3 and online supplemental tables 3–5; combined  $p=0.15$ ,  $0.022$ ,  $0.10$ ,  $0.24$  for time points 0, 30, 60, 120 min, respectively, Fisher's method).

#### Lower levels of short peptides in stool of women with GDM

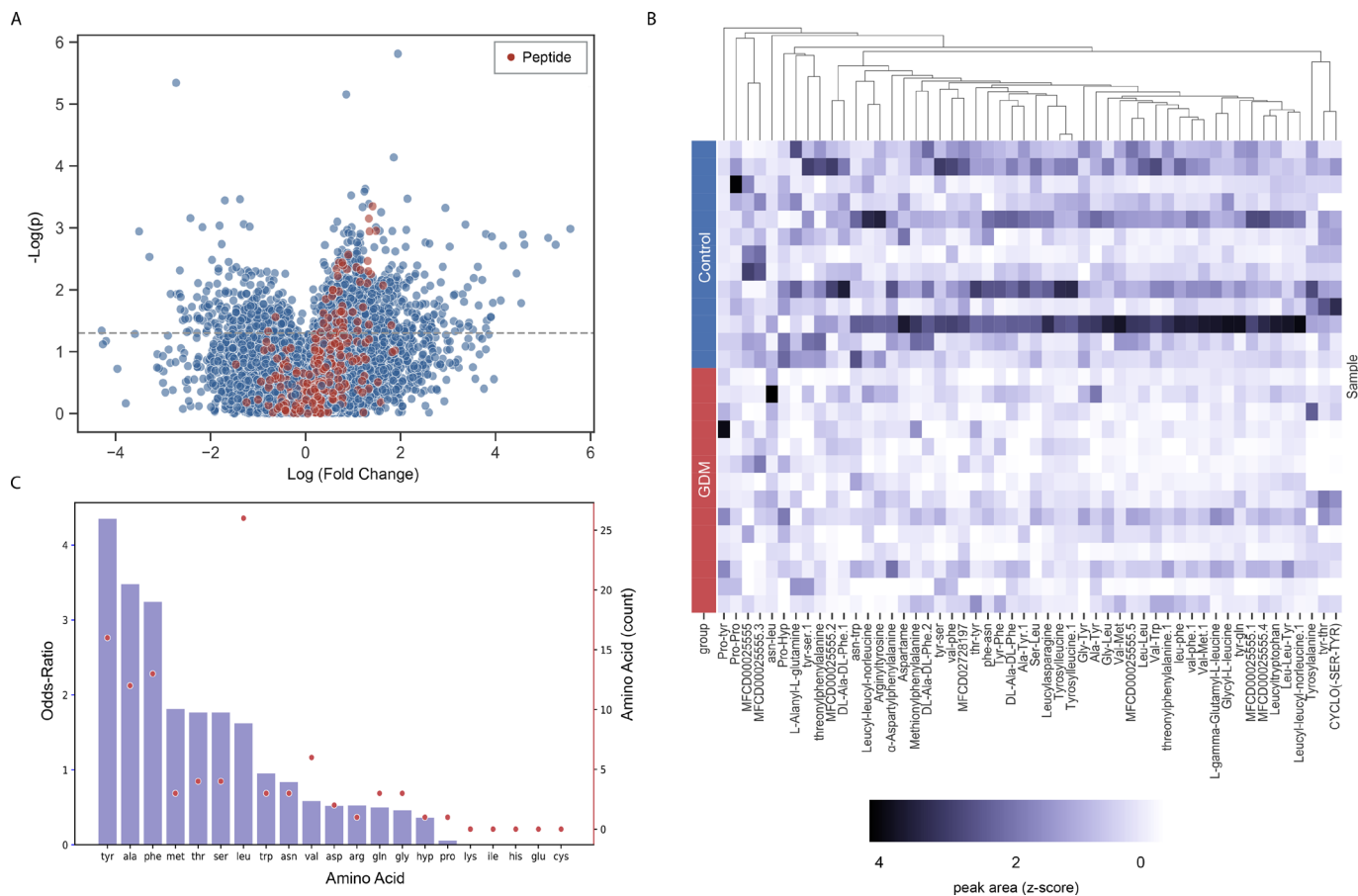
We next compared stool metabolome profiles of women who would and would not later develop GDM ( $n=15$  age-matched and BMI-matched pairs). First, we found a significant correlation between the microbiome and metabolome of these women ( $r=0.26$ ,  $p=0.02$ ; Mantel test). Although we were limited in sample size, manual exploration of the data revealed that many short peptides had differential concentrations (raw  $p\leq 0.05$ )



**Figure 2** Differences in faecal microbiome composition in first trimester between women who would and would not develop GDM later. (A) Principal coordinate analysis based on 16S rRNA gene sequence profiling of the microbiome (GDM: n=28, control: n=236) using the unweighted UniFrac dissimilarity metric coloured by GDM/control (left;  $p=0.06$ , PERMANOVA); violin plots represent the distribution of GDM/control on each axis; Shannon diversity (top right;  $R^2=0.24$  with PCo1) and two phyla that mostly explain the PCo1 and PCo2 variance: Fusobacteria ( $R^2=0.08$  with PCo2) and Deferribacteres ( $R^2=0.3$  with PCo2). (B) The cladogram represents the microbial features associated with the disease state, while controlling for the main risk factors, BMI and age, at all taxonomic ranks. Spearman's rank correlation for each association: a positive association (all associations found), implies over-represented features in the healthy control group. Cladogram and bars are coloured by phylum. BMI, body mass index; GDM, gestational diabetes mellitus; Unc., unclassified; PERMANOVA, permutational multivariate analysis of variance.



**Figure 3** Phenotype transfer via first trimester (T1) FMT to germ-free mice. (A) Study design. (B) PCoA using the unweighted UniFrac metric. Mice receiving FMT from women with GDM exhibit different microbial profiles from mice receiving FMT from the control group ( $p=0.005$ , PERMANOVA test,  $n=7$  age/BMI-matched FMT donor pairs). (C) *Prevotella copri*, which was found to be negatively associated with women with GDM, is negatively associated with GDM-transplanted mice as well ( $p=0.04$ , linear mixed-effects model). (D) Intraperitoneal glucose tolerance test (ipGTT) revealed impaired glucose sensitivity in mice transplanted with faeces from women with GDM in this study and in the Finnish cohort (insert) (error bars represent  $\pm$ SEM;  $*p<0.05$  one-tailed Mann-Whitney U test). (E) Serum cytokine level in transplanted mice ( $*p<0.05$  Mann-Whitney U test). Boxplots indicate the median and IQR; whiskers show IQR $\times$ 1.5. BMI, body mass index; FMT, faecal microbiota transplant; GDM, gestational diabetes mellitus; IL, interleukin; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance.



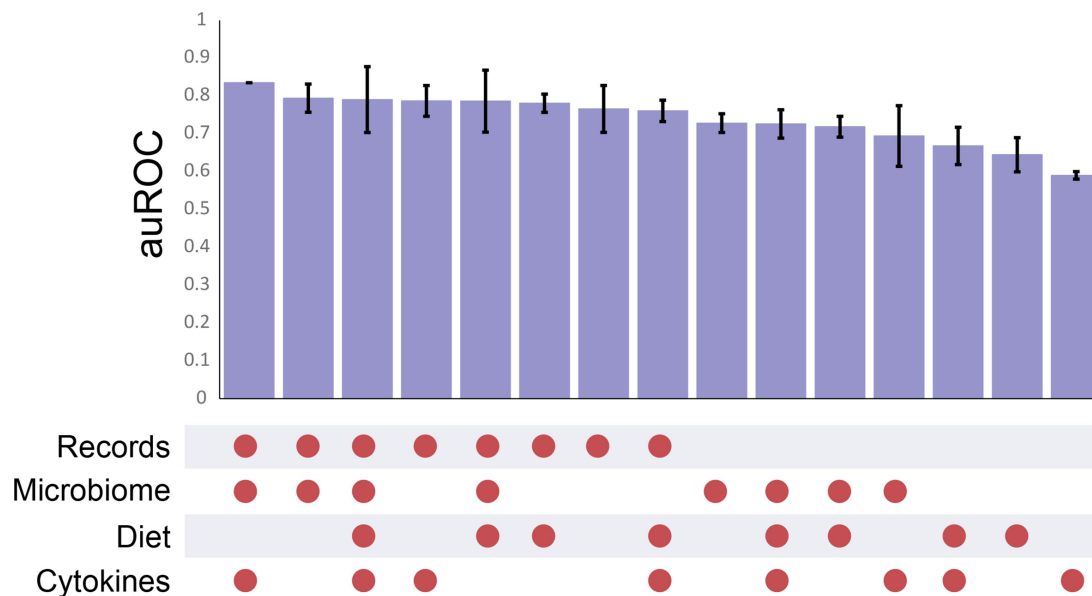
**Figure 4** Analysis of first trimester human faecal metabolomics exhibits lower levels of dipeptides for women with GDM. (A) Volcano plot of all metabolites examined in this study, comparing age/BMI-matched metabolite profiles of women who would and would not later develop GDM ( $n=20$  pairs); peptides are coloured in red. (B) Heatmap of the 52 differentially expressed peptides. Each row denotes a sample (grouped by disease state) and each column denotes a peptide. Z-scores were calculated per column. Peptides (columns) were hierarchically clustered based on Euclidean distances. (C) Amino acid composition of the differentially abundant peptides. Bars (left y-axis) represent odds ratios (OR) for each amino acid, and dots (right y-axis) represent the amino acid count in the differentially abundant peptides. BMI, body mass index; GDM, gestational diabetes mellitus.

between control women and women with GDM (online supplemental table 6). Following curation of all dipeptides and tripeptides, the vast majority of the peptides (50 out of 52) with significant differential concentrations showed a clear tendency of depletion in women with GDM relative to healthy control women (figure 4A,B). These peptides were enriched with the hydrophobic amino acids tyrosine, phenylalanine and alanine ( $p=8 \times 10^{-4}$ , 0.01, 0.01, respectively, FDR-corrected Fisher's exact tests; figure 4C). As metabolome profiling in women uncovered important associations to GDM, we decided to use PICRUSt2 to predict metabolic pathways enriched in mice from the faecal microbiota profiles of the mice in our FMT studies. We found 16 differentially abundant metabolic pathways between GDM and control-recipient mice (online supplemental table 7). We observed an enrichment of the mevalonate pathway (PWY-922; online supplemental figure 4), corresponding with evidence of increased IL-6 levels in the GDM group of both our primary cohort and our transplanted mice<sup>23 24</sup> and of the heme pathway (online supplemental figure 4), previously implicated in type 2 diabetes.<sup>25</sup>

#### Gut microbiome composition improves prediction of GDM early in pregnancy

Finally, we built a machine learning model to predict GDM based on microbiome composition, cytokine profile, medical

history and dietary features, all collected during T1 (figure 1). For this aim, we also included T1 clinical data of 66 additional women, recruited retrospectively in later stages of their pregnancy (secondary cohort, see methods). Our Xgboost model predicted GDM with very high accuracy (area under the receiver operating characteristic curve (auROC)=0.83; figure 5). When making predictions based on only a single feature, we found the highest accuracy when using medical records alone (though still 7% lower than our combined model), in agreement with a recent study.<sup>26</sup> Faecal microbiome features resulted in the second highest accuracy (auROC=0.73). Using our two-step method (see online supplemental methods), we improved the odds ratio (OR) from 3.2 to 4, demonstrating the potential for more accurate prediction using the faecal microbiome profile, especially relevant if medical records are incomplete or unavailable. To validate the predictive power of our microbiome model, we used a validation cohort of 98 women who developed GDM with 98 matched healthy controls from a T1 pregnancy study in China.<sup>8</sup> We first built a model based on the validation cohort to test the predictive power for this cohort based only on microbiome data (auROC=0.65). Assuringly, when applying our model (the Israeli cohort learning set) on this cohort, we found a comparable accuracy (auROC=0.6), confirming that despite the striking genetic and lifestyle differences between the cohorts, our findings are, at least partially, generalisable. We also built a model based on 86



**Figure 5** Highly accurate prediction of future disease onset among pregnant women during their first trimester. Area under the receiver operating characteristic curve (auROC) for each combination of features. Error bars represent  $\pm$ SD.

nutritional characteristics measured in our cohort, which yielded lower predictive accuracy (auROC=0.64; [figure 5](#)) than the other features in this study. Further, no differences were found in dietary habits between women in our primary cohort who would and would not later develop GDM (online supplemental tables 8 and 9) suggesting that differences in food consumption during T1 contribute minimally to GDM pathogenesis.

## DISCUSSION

### GDM biomarkers

Here, using a combination of ‘omics’ tools, we identify biomarkers of GDM onset as early as the first trimester of pregnancy. Women in T1, who later develop GDM, exhibit gut microbiota dysbiosis as well as increased proinflammatory serum cytokines and lower levels of faecal SCFAs. Further, the specific microbial changes in their microbiota are directly associated with GDM phenotype features (insulin resistance and low-grade inflammation) as revealed by FMT into GF animals. Lastly, we demonstrated that microbiota samples from T1 alone can be used to predict GDM onset and that parameters from patient medical records can improve these predictions, providing a robust tool for early prediction of GDM.

In our primary cohort, women with GDM exhibit elevated levels of serum inflammatory cytokines during T1 of pregnancy. Insulin resistance has been associated with elevated secretion of proinflammatory cytokines,<sup>27</sup> and indeed several studies demonstrated elevated levels of proinflammatory cytokines during T2 and T3.<sup>28–30</sup> These altered cytokine profiles in women with several months prior to a GDM diagnosis suggest that inflammation may be associated with the pathogenesis of GDM and can be used to identify its early onset. This is in line with typical GDM and type 2 diabetes symptomatology. Low-grade chronic inflammation is associated with obesity in general and maternal obesity in particular. But here, we controlled for BMI, suggesting that increased pre-GDM-associated inflammation is beyond that associated with obesity or general pregnancy. This is in line with evidence in the literature of inflammation in cases of type 2 diabetes<sup>31,32</sup> independent of weight. Further, in pregnancy, low-grade inflammation levels can differ among women independent

of BMI, suggesting that some other characteristics like immune–endocrine interactions may also be at play. In our study, we specifically observed higher levels of IL-6 in both women with pre-GDM and in GDM-recipient mice. This suggests that the elevated levels of IL-6 are driven by gut microbes. IL-6 was previously described to play a role in the development of both type 1 and type 2 diabetes<sup>33</sup> and was proposed as a potential biomarker of gestational diabetes in 16 different studies, mostly in later stages of pregnancy.<sup>34</sup> Our findings in T1, both in the focal cohort and in FMT experiments, support inflammation as an early marker of GDM.

Another potential early biomarker for GDM is a decrease in SCFAs, which contribute to the maintenance of glucose homeostasis and suppression of inflammatory response. Hence, SCFAs are thought to play a role in obesity-induced inflammation leading to attenuation of insulin signalling and GDM.<sup>35</sup> We found two BSCFAs reduced in stools of women who later developed GDM. BSCFAs are a product of bacterial fermentation of branched amino acids generated from undigested protein reaching the colon. BSCFAs, proposed markers for protein fermentation, were found to improve insulin sensitivity<sup>36,37</sup> and reduce inflammation.<sup>38</sup> These findings, in line with several studies of later-stage pregnancy<sup>39</sup> (but see findings from Pappa *et al*<sup>40</sup>), suggest faecal BSCFAs could serve as a potential biomarker for GDM in early stages of pregnancy.

### The gut microbiome is associated with GDM months before diagnosis

Several studies have found altered gut microbiome composition in women with GDM; most were based on samples collected post-diagnosis.<sup>41,42</sup> Our findings suggest that microbial differences between GDM and control groups, when controlling for confounding variables, exist in T1 and are driven by specific taxa rather than community-wide shifts, leading to subtle differences in composition.

As an illustrative example, *P. copri*, which is known to play a role in glucose homeostasis<sup>43</sup> and has been reported to be more abundant in women diagnosed with GDM,<sup>41,44</sup> was found to be under-represented in women with GDM in our primary cohort,



after controlling for confounders, and also in GDM-recipient mice. A recent study demonstrated that *Prevotella* was a marker of positive glucose metabolism.<sup>45</sup> Kovatcheva-Datchary *et al.* even showed, in a clinical trial, that *Prevotella* protected against *Bacteroides*-induced glucose intolerance and that improvement in glucose metabolism was associated with increased abundance of *Prevotella*.<sup>46</sup> This improved glucose metabolism by presence of *Prevotella* was also demonstrated by supplementing mice with *P. copri*. One possible mechanism, recently proposed in rats, is that *P. copri* improves glucose homeostasis through farnesoid X receptor signalling and increased bile acid metabolism.<sup>47</sup> We chose to discuss *P. copri* specifically as it was found to have lower abundance in both women with GDM and recipient mice and was previously described to play a role in glucose homeostasis. In this study, we also demonstrated the importance of controlling for risk factors. For example, *Akkermansia muciniphila*, which is consistently negatively correlated with obesity,<sup>48</sup> is *prima facie* negatively associated with GDM when not controlling for the difference in BMI between the groups.

### FMT highlights IL-6 as a potential contributor to GDM

Based on our multicohort FMT experiments using T1, pre-GDM-diagnosis samples, we conclude that gut microbes play a causal role in the development of some of the phenotypes of GDM and that their role is likely universal as demonstrated by conservation across cohorts. Increased levels in IL-6, in both women that would develop GDM and transplanted mice that received their microbiota, suggest an important microbiota-related inflammatory mechanism in GDM progression, further supported by functional profile prediction in mice. Two relevant bacterial pathways, the mevalonate pathway and the heme biosynthesis pathway were elevated in GDM-recipient mice. There is evidence that mevalonate can have negative implications on host inflammation—its presence reduces effects of statins in decreasing IL-6 and IL-8,<sup>23</sup> and a kinase deficiency, which increases free mevalonate, leads to autoinflammation.<sup>49</sup> The heme biosynthesis pathway was previously associated with elevated IL-6<sup>50,51</sup> and with type 2 diabetes.<sup>52</sup> We do note, however, that further research is needed to understand if the bacteria themselves, their excreted metabolites or some other factors control this phenotype. Only then can we uncover specific mechanisms of pathogenesis.

### Short peptides association with GDM

We found lower levels of short peptides in T1 stools of women with pre-GDM. These peptides are enriched with the amino acids phenylalanine, alanine and tyrosine. Previously, plasma levels of these three hydrophobic amino acids have also been reported to be significantly associated with diabetes.<sup>53</sup> One study found a link between their elevated blood levels and decreased insulin secretion.<sup>54</sup> Interestingly, Jiang *et al.* recently found elevated levels of alanine and tyrosine in maternal blood at 12–16 gestational weeks in women later diagnosed with GDM<sup>55</sup>; alanine is also used by the liver for gluconeogenesis.<sup>56</sup> Increased amino acid levels in the blood may result in lower levels excreted in stool,<sup>57</sup> though this requires further study.

### Prediction of GDM

We were able to accurately predict future GDM onset in T1, weeks before the complication is typically diagnosed. Our combined model predicts GDM with very high accuracy, and even a microbiota-centric model could predict disease onset in two geographically diverse cohorts. This tool allows for accurate

early prediction, care plans and potential prevention of this disease, improving both maternal and fetal outcomes. This is further supported by phenotype transfer in samples originating from cohorts in three different continents. On the whole, prediction could (and likely should) be improved using local microbiota characteristics, but genus-level differences in the microbiome can be used as general predictors in the absence of local data.

### CONCLUSIONS

In summary, we found broad and consistent evidence that GDM pathology begins as early as T1 in a large prospective cohort of pregnant women. Additionally, we successfully demonstrated that the precursors of GDM originate in the gut microbiota and that early-onset GDM has a bacterial signature at least partially responsible for the GDM phenotype, evident from phenotype transfer following FMT. Our findings suggest that GDM is induced through heightened inflammation, initiated by microbial dysbiosis. Future research based on our findings can help unravel the underlying mechanisms.

This study has several limitations. Bacterial dysbiosis could be a first response to disease onset rather than a cause. Additionally, the phenotype transfer we observed may be caused by other faecal material including metabolites, eukaryotic microorganisms, human viruses and bacteriophages, though in this case as well, the bacterial biomarkers identified can be relevant for diagnostics. Lastly, throughout this study, we treated the major risk factors of GDM, BMI and age, using either matching or relevant statistical methods. We cannot exclude the effect of other clinical or demographic features on our results and also wish to highlight the potentially important contribution of these two ‘confounding’ risk factors. Despite limitations, addition of microbiome data to a machine learning model improved our ability to predict GDM and can even serve as a standalone snapshot predictor. These results may be of use in the future when exploring preventive measures for GDM.

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**Contributors** YP, SF and OK conceived the research. YP, SF and STurjeman analysed the data and wrote the manuscript. SF collected and sequenced the samples and performed experiments. YP, STurjeman, CL, EH, YL and OK wrote the manuscript. AE, MN-O and OZ performed experiments. OS and YL developed prediction models. RS, SK, FM and STamir performed metabolomics experiments. WW, JP, CL, ELJ, SR, SS, EI, KK and REL collected samples and performed experiments of the Finnish or STORK cohorts. EM and EB analysed the data. KT-G, OY, YP, EP, JP, RC, MH and EH supervised sample collection for the primary and secondary cohorts. REL, BS, YL, EH and OK analysed data, provided valuable assets and supervised the research. OK is the guarantor of the research.

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**Patient and public involvement** Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication** Not required.

**Ethics approval** This study involves human participants and was approved by Clalit's Institutional Review Board (approval no. 0135-15-COM) and Rabin Medical Center Institutional Review Board (approval no. 0263-15-RMC). Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** Data are available in a public, open access repositories. All sequencing data were submitted to EBI (project accession number ERP143097). Metabolomics data were deposited at 10.5281/zenodo.6581068.

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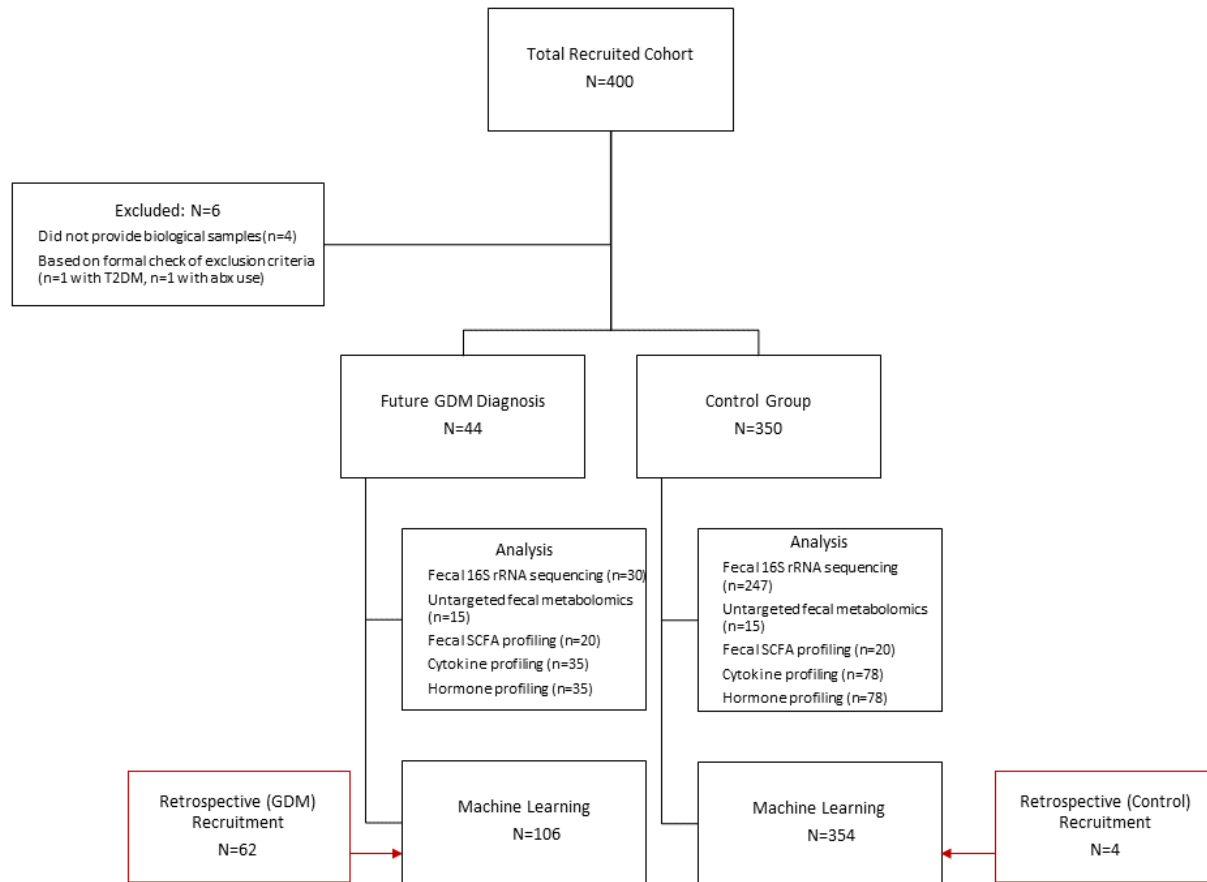
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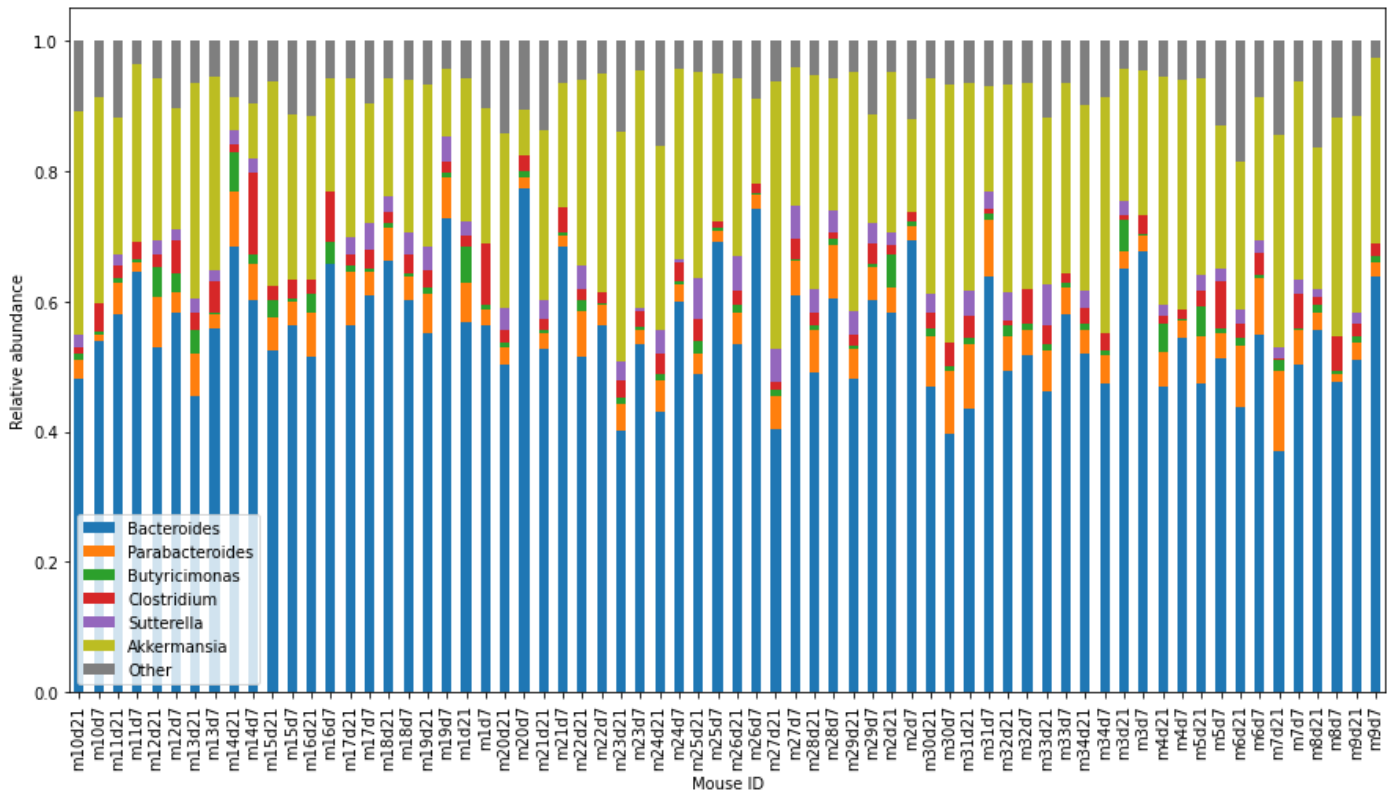
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## **Supplementary Figures for**

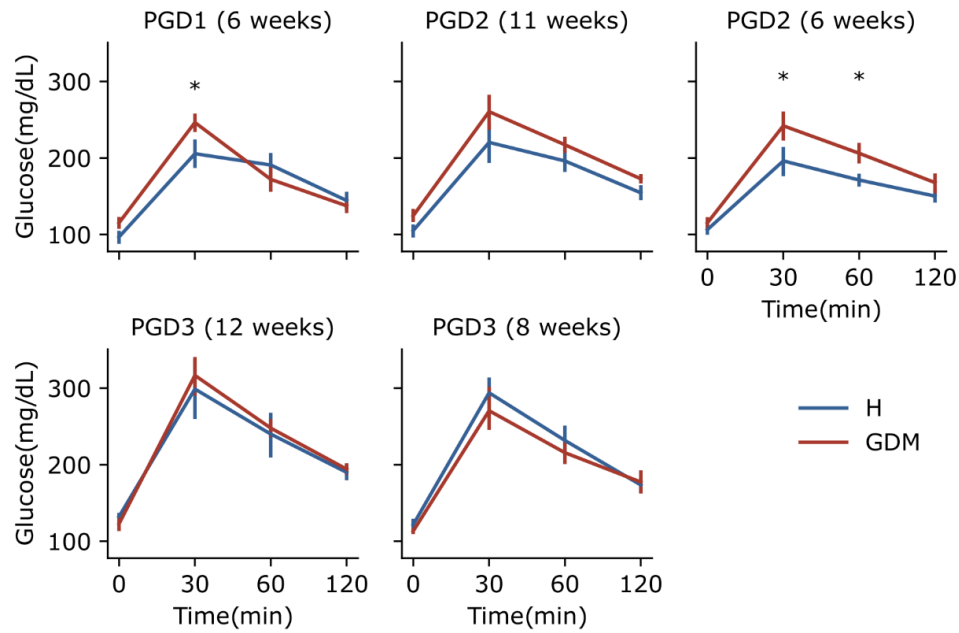
### **Gestational diabetes is driven by microbiota-induced inflammation months before diagnosis**



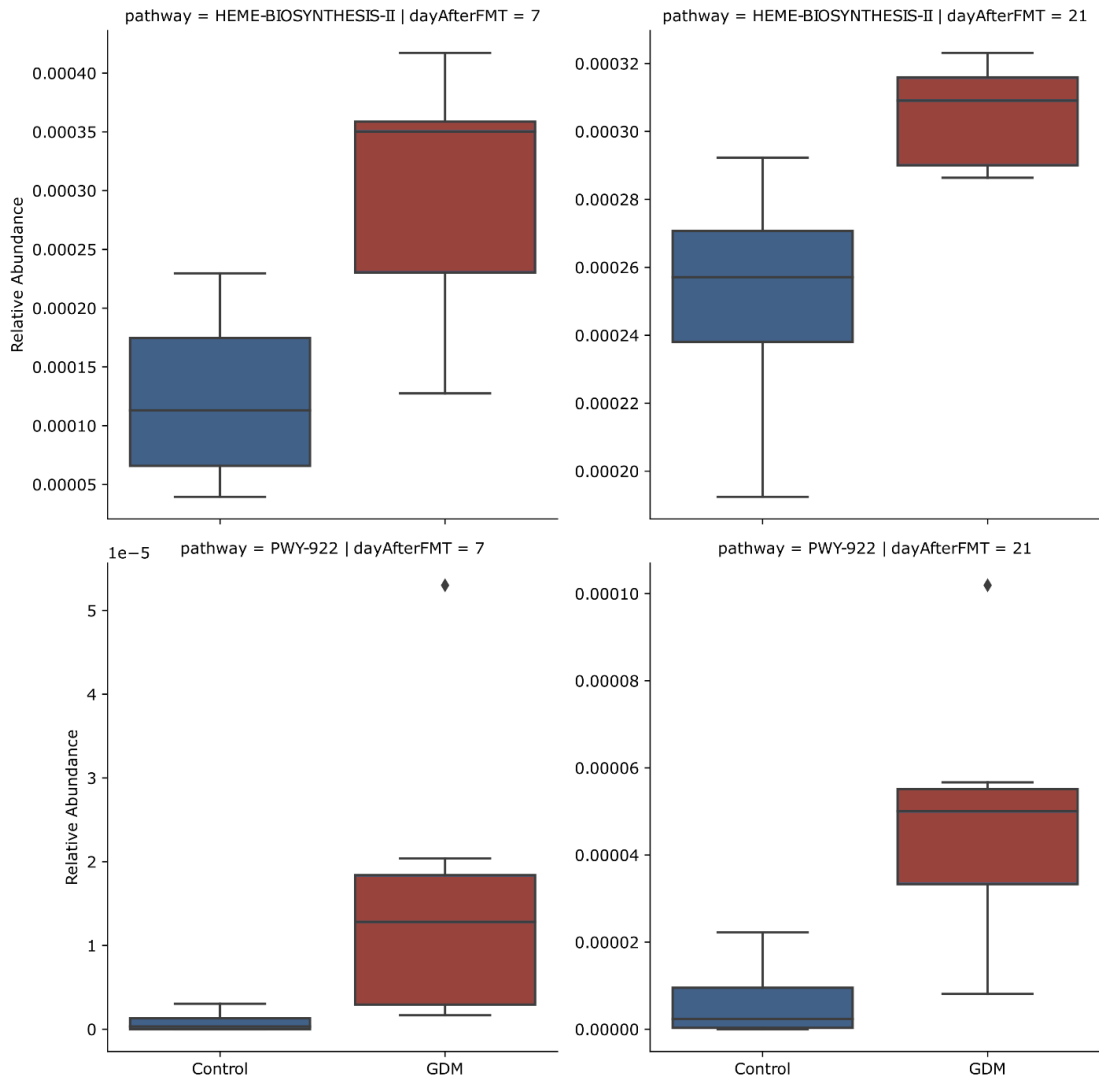
**Supplementary Figure S1.** The study design for the multi-omics and prediction modeling arms of this research. Initially, a Primary Cohort of 400 women was recruited prospectively and 394 were included in the study - 44 who would go on to develop GDM and 350 who would not (the “healthy control group”). To increase power in our predictive models, we recruited a Secondary Cohort retrospectively which included 62 women with GDM and 4 without. Clinical data from their first trimester of pregnancy was retroactively extracted from the medical records following their recruitment in their second trimester of pregnancy (after GDM status was already known).



**Supplementary Figure S2.** Relative abundances of transplanted mice at the genus level. Columns are labeled by the mouse number and day after transplantation (e.g. m1d7 is mouse number 1, 7 days post transplantation).



**Supplementary Figure S3.** intraperitoneal glucose tolerance test (ipGTT) exhibit impaired glucose sensitivity in mice transplanted with feces from GDM women in the Finnish cohort (upper row) and in the American STORK cohort (lower row). \* $p < 0.05$  Mann Whitney U test. Combined  $p = 0.15, 0.022, 0.10, 0.24$  for timepoints 0, 30, 60, 120 minutes respectively, Fisher's method.



**Supplementary Figure S4.** Relative abundances of the MetaCyc heme pathway (up) and mevalonate pathway (down) as predicted by PICTUS<sub>t</sub>2 for the transplanted mice, 7 days post transplantation (left) or 21 days post transplantation (right). FDR corrected  $p < 0.05$ , linear mixed model (see methods).



## Supplementary Methods

### Contents

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  - o FMT for additional cohorts
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### Primary cohort sample collection

Blood samples (15ml) were collected in EDTA tubes from the primary cohort at recruitment. The tubes were centrifuged at 4°C for 10 min at 3600 rpm; plasma was then stored in coded 1.7 ml tubes at -80°C until processing. Fecal samples from this cohort were collected as previously published [1], from participants at 11-14 gestational weeks, close to recruitment, and frozen immediately at -80°C until processing. These samples were used to profile the gut microbiome composition, short-chain fatty acids (SCFAs) and the metabolome.

### GDM diagnosis method

In Israel, GDM diagnosis is done with the 2-step procedure [2]: glucose challenge test (GCT) and an oral glucose tolerance test (OGTT). GCT screening is universal at 24-28 weeks and followed by a diagnostic OGTT if GCT>140mg/dl. Sometimes for women with risk factors (BMI, FPG, Past GDM) the OGTT, without GCT, is done late in the first trimester. In our study no participants underwent this testing prior to providing samples. Exact timing of GDM diagnosis does not affect the

findings of our study, and our microbiota, metabolome, and inflammation markers provide an earlier window into disease risk than current diagnostic practices.

### **Additional cohorts**

In addition to the primary and secondary cohorts, for fecal microbiome transplant (FMT) experiments in germ-free (GF) models, described below, fecal samples from pregnant women in T1 who would and would not go on to develop GDM from two additional independent cohorts were included: samples from a Finnish cohort [3–5] and the Stanford Outcomes Research in Kids (STORK) study [6]. The subjects in the Finnish cohort were recruited during the first trimester of pregnancy to a randomized, controlled trial (ClinicalTrials NCT00167700) assessing the impact of dietary counseling and a probiotic intervention on various maternal and infant outcomes [3–5]. Pregnant women with chronic disease including metabolic abnormalities were excluded from the study. Fecal samples were collected at recruitment during T1 prior to intervention, and again during the third trimester (T3; the latter were not used in this study). Consequently, T1 samples were collected from metabolically healthy women. GDM was diagnosed by a two-hour OGTT, which was performed at 24–28 weeks of gestation in subjects at increased risk including women with excessive weight gain, age >40 years, glucosuria, increased fetal growth or a history of GDM or macrosomic newborn(s) in previous pregnancies. GDM was diagnosed if plasma glucose concentration was  $\geq 4.8$  mmol/L at baseline,  $\geq 10.0$  mmol/L at 1 h or  $\geq 8.7$  mmol/L at 2 h during the OGTT. The STORK study is a multiethnic birth cohort from California [6]; for this study, a total of 6 mothers diagnosed with GDM in T1 were matched on gestational age, maternal age and race/ethnicity to 6 controls with fecal samples collected between 10.5 to 23 (mean 16) weeks of pregnancy.

### **Serology: primary cohort**

Cytokine (TNF- $\alpha$ , IFN- $\gamma$ , GM-CSF, IL-2, IL-4, IL-6, IL-8, IL-10) and hormone (insulin and leptin) levels were measured in plasma using the Bio-Plex Pro Human Cytokine 8-Plex Panel (Bio-Rad Laboratories Inc., Irvine, CA, USA) according to the manufacturer's instructions. Serum was not available for all women in the GDM group such that 35 of the 44 pre-GDM women (and 78 controls) were included in this analysis.

### **Microbiome sequencing and pre-processing**

DNA was extracted using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions and following a 2-minute bead beating step (BioSpec, Bartlesville, OK, USA). Purified DNA was used for PCR amplification of the variable V4 region using the 515F and 806R barcoded primers following the Earth Microbiome Project protocol [7]. For each PCR reaction, the following materials were added: 4 µl (~40ng/ µl) DNA (sample), 2 µl 515F (forward, 10 µM) primer, 2 µl 806R (reverse, 10 µM) primer, and 25 µl PrimeSTAR Max PCR Readymix (Takara, Mountain View, CA, USA). PCR reactions were carried out by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 5 seconds, extension at 72°C for 20 seconds and then a final elongation at 72°C for 1 minute. Amplicons were purified using AMPure magnetic beads (Beckman Coulter, Brea, CA, USA) and quantified using the Picogreen dsDNA quantitation kit (Invitrogen, Carlsbad, CA, USA). Then, equimolar amounts of DNA from individual samples were pooled and sequenced using the Illumina MiSeq platform and MiSeq Reagent Kit V2 (500 cycles) at the Genomic Center at the Bar-Ilan University Azrieli Faculty of Medicine, Israel.

Microbial diversity and composition were assessed using QIIME2 version 2019.4 [8]. First, single-end sequences were imported (`qiime import`) and demultiplexed (`qiime demux`) with golay error correction. Next, sequences were denoised using DADA2 [9] (`qiime dada2 denoise-single`), trimming the first 5 bases and truncating each sequence at position 215. Feature tables and representative sequences from the different sequencing runs were then merged. A phylogenetic tree was constructed using the fragment-insertion method (`qiime fragment-insertion sepp` [10]). Taxonomic classification was done using a naïve-based classifier trained on the 99% Greengenes 13\_8 V4 reference set [11] (`qiime feature-classifier classify-sklearn` [12]). In order to remove low-confidence features, only features with a frequency higher than 50 in at least 5 samples were kept. In addition, features that contained mitochondria or chloroplast sequences or that were not assigned to a phylum were filtered out. Data were then rarefied to 8,000 (human) or 16,000 (mouse) sequences per sample (`qiime diversity core-metrics-phylogenetic`).

### **Short-chain fatty acids profiling**

Short chain fatty acid (SCFA) extraction and analysis was performed at the MIGAL Galilee Research Institute, Israel. An aliquot of 0.25 gr of wet feces from 20

age matched pre-GDM and control pairs was thawed and suspended in 1 ml of an orthophosphoric acid solution (8% v/v) and kept at room temperature for 10 min with occasional shaking. The mixture was homogenized for 2 min, and the suspension was centrifuged at 4°C for 15 min at 14,000 rpm. The supernatant was filtered by additional centrifugation at 4°C for 15 min at 14,000 rpm. Next, 225 µl of the supernatant were transferred into a polypropylene tube, and 25 µl of 2-methyl-butyric-acid (Sigma-Aldrich (Merck), St. Louis, MO, USA) were added as an internal standard (IS) to a final concentration of 0.001M and transferred to a chromatographic vial for gas chromatography analyses. The IS was used to correct for injection variability between samples and for minor changes in the instrument response. Vials were stored at -20°C before GC analysis. A standard mix (WSFA-4, Sigma-Aldrich, St. Louis, MO, USA) was used to determine the concentrations of propionic acid. Standard curves for acetic acid and butyric acid (Sigma-Aldrich, St. Louis, MO, USA) were prepared using stock solutions of both acids, separately.

Gas chromatography analysis was then performed. Chromatographic analyses were carried out using the Agilent Technologies 6890, a GC system with a mass selective detector. A fused-silica capillary column with a free fatty acid phase (DB-FFAP 122-3232, 30 m×0.25 mm×0.25 µm) was used. The carrier gas was helium at a flow rate of 13.6 mL/min. The initial oven temperature was 70°C, raised to 100°C at a rate of 20°C/min, then raised to 180°C at 8°C/min and held for 3 min, before then being raised to 230°C at 20°C/min. The injection volume was 1 µL and the run time of a single analysis was 17 min.

### **Untargeted metabolomics**

Untargeted metabolomics was performed at the MIGAL Galilee Research Institute, Israel and Tel Hai College, Israel on fecal samples from 15 pairs of BMI- and age-matched women who would and would not go on to develop GDM. Fecal samples were extracted using methanol (0.333 mg/ml of MeOH), vortexed, and centrifuged. The supernatant was collected and filtered before injection to the LC-MS/MS instrument. A pooled matrix prepared by mixing a small volume (20 µl) of each experimental sample was used as a quality control (QC) for batch normalization and compound identification.

The samples were injected (5 µL) into UHPLC connected to a photodiode array detector (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA), with

a reverse-phase column (ZORBAX Eclipse Plus C18; Agilent, Santa Clara, CA, USA; 100\*3.0 mm; 1.8  $\mu$ m). The mobile phase consisted of (A) DDW with 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The gradient was initiated with 2% B which was increased to 30% B over 4 min, and then increased to 40% B over 1 min before being kept isocratic at 40% B for another 3 min. Then, the gradient increased to 50% over 6 min, and to 55% over another 4 min and to 95% over 5 min and kept isocratic for 7 min. Finally phase B was returned to 2% over 3 min and the column was allowed to equilibrate at 2% B for 3 min before the next injection. The flow rate was 0.4 mL/min. Blank (methanol) and QC samples were injected at the start of the sequence, after every 10 samples, and at the end of the sequence.

LC–MS/MS analysis was performed with a Heated Electrospray ionization (HESI-II) source connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Scientific™, Germany. ESI capillary voltage was set to 3500 V, capillary temperature to 300°C, gas temperature to 350°C and gas flow to 10 mL/min. The mass spectra ( $m/z$  100–1500) were acquired using both positive and negative ion modes. Data dependent MS2 analysis was generated for the QC samples and used for compound identification. Downstream analysis and data processing were performed with the Thermo Scientific™ Compound Discoverer™ program, version 3.1.0.305 (mass tolerance  $\leq$  5ppm; intensity tolerance  $\leq$  30%; S/N threshold = 3; minimum peak intensity = 1,000,000; RT tolerance  $\leq$  0.2min). Databases used for identification were Chempider [13], MzCloud [14] and KEGG [15].

### **FMT into germ-free mice**

Transplantation experiments were performed using fecal samples from the primary prospective cohort and the two additional cohorts (described in the supplementary methods). First, for samples from the primary cohort, we used the model of fecal gut microbiome transplants to germ-free mice as conducted previously [1,16,17]. Briefly, germ-free (GF) female Swiss Webster mice (8 weeks old for the Israeli cohort; other FMT experiments detailed below) were maintained in isolators under a strict 12h light:12h dark cycle with estrous cycles synchronized to minimize mouse hormonal variation. Mice were fed an autoclaved chow diet (Harlan-Teklad, Madison, WI) ad libitum. Stool samples from T1 pregnant women who were and were not later diagnosed with GDM were selected based on age- and BMI-matching without a priori knowledge of bacterial diversity. Fecal matter (0.1 g) was

suspended in 1.5 ml of reduced sterile PBS, vortexed for 5 min and settled for 5 min to allow larger particles to settle to the bottom of the tube. Handling of human fecal samples was performed under anaerobic conditions. Mice were divided into two groups with equal weights and then immediately gavaged with 200  $\mu$ l of fecal slurries from the 2 study groups. Each fecal slurry was gavaged into a single mouse and the mice were then placed in ventilated cages, 3-4 mice per cage (divided by treatment group) and followed for 4 weeks.

Body weight and chow consumption were monitored weekly. Fecal pellets were collected on days 7, 14 and 21, snap-frozen in liquid N<sub>2</sub> and stored at -80°C for analysis of microbial communities. On day 21, intraperitoneal glucose tolerance test (ipGTT) was performed by an injection of 2 g/kg body weight glucose after an 8 h fast. Tail blood samples were collected at 0, 15, 30, 60, 90, and 120 minutes and blood glucose levels determined. On day 29, mice were sacrificed, and blood samples and ceca were collected.

#### *FMT for additional cohorts*

FMT experiments using samples from the additional cohorts were performed in the Cornell University animal facility. Deviations from the above protocol, used with primary cohort samples, are outlined here: For the Finnish cohort, two sets of experiments were performed. PGD1 study: stool samples were obtained in T1 from 6 women diagnosed with GDM matched to 6 healthy controls from Finland [18]. Twelve 6-8 week old female GF Swiss Webster mice were gavaged with stool sample slurries prepared under anoxic conditions as previously described. Here, an OGTT was administered 12 days post inoculation. Glucose dose was 2g/kg; readings were at 0, 30, 60, 120 minutes, via ACCU CHEK Compact Plus (Roche, Mannheim, Germany). PGD2: repeat of PGD1 with 12 mice aged 6-8 weeks and 12 mice aged 11-13 week old mice (total of 48). OGTT was performed on day 19.

For the STORK cohort, PGD3, 8 week old mice and 12 week old mice were gavaged with stool collected on gestational age of 16 weeks. As above, each donor sample was inoculated into one 8 week old and one 12 week old female mouse. An OGTT was administered 12 days post inoculation. Glucose dose was 2g/kg; readings were at 0, 30, 60, 120 minutes, as above.

#### **Serology: FMT with primary cohort samples**

Mouse cytokine (IL-1b, IL-6, IL-10, IL-17A, IFN- $\gamma$ , TNF- $\alpha$ ) and hormone (insulin and leptin) levels were measured in plasma (see experimental design below) of mice transplanted with samples from the primary cohort using the BioPlex Mouse Cytokine 8-plex Immunoassay (Bio-Rad Laboratories Inc., Irvine, CA, USA) according to the manufacturer's instructions. The fluorescent signals were measured on a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories Inc., Irvine, CA, USA). Analyte concentrations were calculated using standard curves in the Bio-Plex Manager Software. Values out of range (below/above) were imputed with the minimal/maximal in range values respectively.

### **Prediction**

To predict GDM, we developed a prediction model using our prospective cohort based on all T1 information. We checked each combination of the following components: 1) cytokines, 2) microbiome, 3) general clinical information and 4) food questionnaires (15 total combinations). The accuracy of the prediction was assessed using the Area Under Curve (AUC) of the test set, in a 20%/80% test/training set division and a five fold cross validation.

The microbiome was merged into a genus level representation, log transformed and merged using the standard parameters of the MIPMLP pipeline [19]. For the other components, all non-numerical values were replaced by a one-hot representation. All missing values were replaced by the median value as of the same category. All values were z-scored to an average of 0 and a standard deviation of 1.

We used a binary XGBoost [[20] with a learning rate of 0.001, 200 estimators, gblinear classifiers, a logistic loss function, a lambda regularization of 0.01, and gamma regularization of 0.1 with the XGBclassifier function. All other parameters were the default of the function. The binary outcome was whether the woman later developed GDM. When combining different types of inputs for the classification, the inputs were concatenated.

We limited the external feature analysis to features informative on the training set in the first cross validation (Pearson correlation in the training test with the outcome of p value <0.1). The resulting feature used were:

Smoking (Yes/No/Past); BMI; FGT T1 (fasting glucose test, 1st trimester); Sleeping hours T1; GOT\_T1 (serum glutamic-oxaloacetic transaminase); GPT T1 (serum

glutamate-pyruvate transaminase); PAPP-A[mU/L](Pregnancy-associated plasma protein A); Aspirin T1 (binary); medications\_T1 (chronic medications).

When performing a two-step approach, we first predicted GDM using only the external features above. We then performed a microbiome only classification on the entire test set of the first stage classification. This resulted in two scores. One for the clinical features and one for the microbiome, further denoted: S(Clinical) and S(Microbiome). Samples with a low S(Clinical) or S(Microbiome) value were defined as negative. Samples with high scores for both S(Clinical) and S(Microbiome) values were defined as positive.

To examine the generalizability of our model, we applied the XGBoost classifier to an independently published 16S rRNA dataset from a cohort in China [21]. Briefly, this cohort included 98 pairs of pregnant women with and without GDM (matched) that provided a fecal sample in week 10-15 of pregnancy. We applied the same hyperparameters that were used on the primary cohort. The processing of the microbial data was similar to that mentioned above. Further, we built a model based on the intersection of bacteria between the two cohorts (67 shared microbes at the genus level). We trained the model on the main cohort and tested the model performance on the Chinese cohort. To improve the performance of the transfer learning, we used the iMic model [22]. The iMic model uses the taxonomy structure of the microbiome to translate the microbiome into images. Then convolutional neural networks (CNNs) are applied to the images. For the cross validity with iMic, we used the data at the species level.

### Statistical analysis

Demographic and lifestyle characteristics of women with and without GDM were compared using t-tests or Fisher's Exact tests as appropriate; serum levels of cytokines and hormones, concentrations of short chain fatty acids were compared using the non-parametric Mann-Whitney U test followed by FDR correction as implemented in the scipy stats library of python [23].

Microbiome data was preprocessed (see above) and then diversity analysis was performed. Differences in alpha-diversity (Shannon's diversity index) were tested using a Kruskal-Wallis test (implemented in qiime diversity alpha-group-significance). Un/weighted UniFrac [24], Bray Curtis and Jaccard were used as metrics of paired distance between samples (beta-diversity), and the permutation-based PERMANOVA



test was performed (qiime diversity beta-group-significance) to test whether distances between samples within a group (GDM/control), were more similar to each other than they were to samples from the other group. To incorporate the major risk factors (BMI and age) into the model, we fit a distance matrix and used diagnosis, BMI and age as explanatory variables using `adonis2` [25,26]. Since the model inputs variables sequentially, we fit 6 different models to examine all of the different orders of the variables.

Mantel's correlation between features were performed and unweighted UniFrac distances was used as the metric for microbial dissimilarity. For all other features, data was log transformed and min-max normalized, and Euclidean distance was used as the distance metric. 9999 permutations of label mixing were done; the p-value was calculated as the proportion of these permutations that lead to a higher explained variance than the measured explained variance.

To associate microbial features with GDM, features were collapsed to the different taxonomic levels from phylum to species. Spearman rank correlations were used to identify associations between the disease state for each microbial feature at each taxonomic level. Disease state labels were mixed 1000 times to receive a background distribution, and only correlations with  $p < 0.01$  were preserved. To control for the main risk factors of GDM, age and BMI, we adjusted associations by building a linear model and performed Spearman rank correlations on the linear regression residuals. Specifically, we regressed the disease state label over the age and BMI and computed the residual. We then computed the correlation between the different bacteria and the residual and compared that with the results obtained when scrambling the residuals among patients.

When considering the fecal microbiota profiles from the FMT study, microbial features were associated with GDM donors using `MaAsLin2` [27] to perform per feature linear mixed effects (LME) modeling (see supplementary methods). Features were first log transformed and were subjected to cumulative sum scaled (CSS) normalization. Disease state (GDM/control) and days-post-FMT were used as fixed effects while cage and donor were included as random effects following Eq.1.

$$\text{Eq 1: feature} \sim \text{disease} + \text{days\_after\_fmt} + (1 \mid \text{cage}) + (1 \mid \text{donor}).$$

MetaCyc pathway abundances per sample were predicted using PICRUSt2 [28] with default parameters and using DADA2 amplicon sequence variants as inputs. Pathways with more than 25% zeros across samples were

removed. We next applied LME models (Eq. 2) to identify differentially abundant pathways between mice with FMT from women with vs. without a later diagnosis of GDM. Finally, we used Wald chi-square tests to determine the significance of the 'disease' fixed effect in each model and applied FDR corrections to all generated p-values.

Eq 2: pathway ~ disease + days\_after\_fmt + (1 | mice\_ID)

For untargeted metabolomics, differential abundances of the metabolites between the groups were identified by log transformation of the peak areas followed by student's t-tests and FDR correction. Short peptides were manually curated using the metabolite name and using a list of dipeptides downloaded from the PubChem database [29]. Enrichment of amino acids was calculated using Fisher's exact test with the following contingency table groups: amino acid of interest, all other amino acids, peptides enriched in GDM, peptides not enriched in GDM.

### **Ethics statement**

Informed consent was obtained from all participants of the main and secondary cohorts in accordance with Clalit's institutional review board approval No.0135-15-COM for the main cohort and with Rabin Medical Center institutional review board approval No.0263-15-RMC for the secondary cohort.

All experiments involving mice were performed using protocols approved by the local animal ethics committee at Bar-Ilan University (number 33-04-2018) and the IRB at Cornell University (Number NCT00167700). The STORK subjects were approved under Stanford IRB protocol number 17756.

### **Patient and public involvement**

Participants were not involved in developing the research question nor the design of this study. Main and secondary cohort members were first involved when they were enrolled; their informed consent included time required to participate and no expectation of involvement in the dissemination of results.

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