Gastro Hep Advances 2022;1:993–1005

ORIGINAL RESEARCH—BASIC

Urine and Serum Metabolomic Profiles Differ by Disease Activity in Pregnant Women With Inflammatory Bowel Diseases

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BACKGROUND AND AIMS: Inflammatory bowel disease (IBD), inclusive of ulcerative colitis and Crohn's disease, are chronic inflammatory conditions that impact women of childbearing age. It has been previously shown that IBD is associated with altered metabolomic profiles, but whether metabolomic changes also affect pregnant patients with IBD is completely unknown. **METHODS:** This was a prospective cohort study comprised of 48 pregnant women with IBD who were followed throughout preconception and pregnancy. IBD disease activity was measured using biochemical markers C-reactive protein or fecal calprotectin using enzyme-linked immunosorbent assay and clinical disease activity using Harvey-Bradshaw Index or partial Mayo scores. Serum and urine samples were collected from preconception, trimester 1, and trimester 2 and analyzed using nuclear magnetic resonance spectroscopy combined with metabolomics set enrichment analysis. **RESULTS:** We identified a total of 24 urine metabolites and 17 serum metabolites which were altered by active disease across pregnancy. First trimester (T1) active disease-associated metabolites were enriched in "amino acid metabolism" and "fatty-acid β-oxidation." The leading urine metabolites at T1 were trimethyl-N-oxide (TMAO), succinic acid, and 3-hydroxy-2-methylbutyric acid, and leading serum metabolites were TMAO, glucose, and acetic acid. Multivariate modeling using serum TMAO, glucose, and acetic acid predicts T1 disease activity and correlated with mode of delivery and infant weights at delivery. Moreover, cross–time point modeling using metabolomes predicted future disease flare-up during pregnancy. **CONCLUSION:** These results suggest select host metabolites may be able to discriminate and predict disease activity and are correlated with pregnancy outcomes at delivery. This warrants further validation of metabolomics to monitor IBD in pregnancy.

Keywords: IBD; Pregnancy; TMAO; Metabolomics

Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapse-remitting inflammatory conditions that involve the gastrointestinal tract.[1,2] IBD often presents early in adulthood and therefore impacts a significant portion of women in their childbearing years.[1] Women with IBD and specifically those with active inflammation during pregnancy tend to have worse pregnancy outcomes including prematurity, cesarean deliveries, and low-birth-weight infants.[3,4] For this reason, women contemplating pregnancy are currently recommended to conceive during remission,[1] and strategies to detect and monitor IBD activity during pregnancy are crucial to improving pregnancy outcomes.

While endoscopic evaluation remains the cornerstone of assessing disease activity, the risk of hemodynamic instability to fetus,[5] preterm labors,[5] and miscarriages[6] preclude the use of serial endoscopy in patients during pregnancy.[7,8] On the other hand, clinical tools such as Harvey-Bradshaw Index (HBI) and partial Mayo scores (pMayo) may circumvent the potential risks to the fetus, but they do not necessarily correlate with inflammation in pregnancy.[9] As such, there has been a focus on noninvasive biomarkers, with examples including C-reactive protein (CRP) and fecal calprotectin (FCP) which are 2 validated biomarkers with strong correlation with endoscopic and histologic disease progression.[10–12] Yet despite their predictive role in disease activity, CRP levels fluctuate throughout pregnancy,[13] and FCP is known to suffer from significant diurnal and intra-individual variations and varies by testing kits.[14] As such,

Abbreviations used in this paper: **AUC, area under the curve; CD, Crohn's disease; CI, confidence interval; CRP, C-reactive protein; FC, fold change; FCP, fecal calprotectin; HBI, Harvey-Bradshaw Index; IBD, inflammatory bowel disease; IQR, interquartile range; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least squares–discriminant analysis; pMayo, partial Mayo scores; TMAO, trimethylamine-N-oxide; UC, ulcerative colitis; VIP, variable important in projection.**

Most current article

2772-5723
https://doi.org/10.1016/j.gastha.2022.07.008

the need of a single noninvasive discriminatory test to distinguish active disease states during pregnancy cannot be more overstated.

Metabolomics is an exciting area that focuses on the systemic quantification of metabolites in biological samples.[15] Like other "-omic" approaches, metabolomics focuses on systems biology and has the advantage of detecting earlier changes,[16] unmasking relevant disease networks and mechanisms,[17] and permits noninvasiveness and versatility in clinical sampling, including fecal extracts, urine, and blood.[18] Previously, it has been shown that IBD was associated with altered urine and serum metabolite profiles,[19–21] and select metabolites in urine samples were able to distinguish active IBD vs remission using nuclear magnetic resonance (NMR) spectroscopy.[21] However, no studies to date have examined if (a) metabolomic changes also occur during active disease in pregnancy and if (b) metabolite changes have any correlations with clinical outcomes on either IBD or obstetrical outcomes.

To delineate the effects of disease activity on maternal metabolomics, our objective was to measure the urine and serum metabolite profiles using NMR spectroscopy of pregnant women with IBD. Furthermore, to understand their clinical correlations, we performed metabolite set enrichment analysis (MSEA) to identify predictive biomarkers and interrogated their clinical correlations with maternal disease activity across pregnancy and future obstetrical outcomes.

Materials and methods

Ethical Statement for Human Studies and Subjects

This was a single-center prospective cohort study performed at the University of Alberta (Edmonton, Alberta, Canada) between 2014 and 2017. The study design was approved by the institutional health research ethics board (Pr000056685). All participants provided written informed consent and were excluded if below the age of 18 or were unable to provide consent to participate within the study. Each participant's collected data and samples were de-identified using standards outlined by the Health Insurance Portability and Accountability Act Safe Harbor section for removal of identifiers.

Data Sharing and Data Accessibility

The data underlying this article cannot be shared publicly due to reasons of patient confidentiality for participants involved in the study. Data sharing will be considered for reasonable requests to the corresponding author in discussion with affiliated institutions.

Cohort Selection and Baseline Visits

Participants were included if they have a diagnosis of UC or CD. They were excluded if they had a history of indeterminate colitis, surgical interventions (ileostomy, pouch or bowel resections), and autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis). Baseline demographics were collected at the initial baseline visit at the pregnancy clinic at the time of recruitment. These included age at study inclusion, age of diagnosis, ethnicity, education, marital status, IBD subtype (UC or CD), disease phenotype, localization of disease, and previous medication use.

Patient Follow-Up

Following baseline visits, all participants were followed at the ambulatory pregnancy IBD clinic at trimester 1 (0–13 weeks and 6 days), trimester 2 (14–26 weeks and 6 days), and trimester 3 (27 weeks to delivery). During each visit, clinical information was collected including current IBD and non-IBD medications, hospital admissions and IBD disease activity assessments using clinical disease indices (pMayo and modified HBI), and serum CRP or maternal FCP. Clinically active disease was defined as modified HBI $\geq$5 or pMayo $\geq$2, and biochemically active disease was defined as FCP $\geq$250 μg/g or CRP $\geq$10 mg/l. In addition, the use of nutritional supplements (multivitamins, folate, iron, omega-3 fatty acids, cod liver oil, calcium, and vitamin D supplementations) was recorded prospectively at each trimester.

Maternal Sample Collection and Processing

Single urine and serum samples were collected at follow-up hospital visits in preconception, trimester 1, and trimester 2. Participants were given collection containers and instructions for collection. Urine samples were collected in sterile bottles with 0.02% weight/volume of sodium azide and stored at −80 °C until NMR spectroscopy. All samples collected were processed and analyzed for NMR spectroscopy at The Metabolomics Innovation Centre (University of Alberta, Edmonton, Alberta, Canada) using methods previously described.[19,22] Briefly, urine samples were centrifuged, and 65 μL of supernatant was mixed with 585 μL of internal standard (Chemox Inc, Edmonton, Alberta, Canada). The pH was adjusted to 6.8 $\pm$ 0.1 using NaOH or HCl. Venipuncture blood samples were obtained from patients during the same follow-up visits. At collection, samples were incubated at room temperature and centrifuged to extract serum and stored at −80 °C until NMR spectroscopy. Serum processing was performed as previously described.[22] In brief, serum samples were thawed and proteins were deproteinized using ultrafiltration. Glycerol and macromolecules were removed from the serum samples by washing and centrifuging serum samples through a 3 kDa filter (10,000*g*, 30 min). Final samples were added with D_2O and standard buffer solution as previously described.[19] All processed samples were stored at −80 °C until NMR spectroscopy.

NMR Spectroscopy

Processed urine and serum samples were thawed at room temperature and were assayed based on previously described protocols by The Metabolomics Innovation Centre.[23] ^{1}H-NMR spectra were analyzed using the Chenomx NMR Suite Professional software package (version 7.6; Chenomx Inc). Quantitative analysis was done using internal standard for chemical shift references (0 ppm) and metabolite quantification. Quantification accuracy was previously verified elsewhere.[19]

Data Processing and MSEA

Raw serum and urine metabolite NMR concentration data sets were normalized using methods previously described[17,19] and

analyzed for MSEA using MetaboAnalyst 5.0.[24] In brief, urine metabolite concentrations were normalized against creatinine, nontransformed, and autoscaled (also known as unit variance scaling). Serum metabolite concentrations were nontransformed and autoscaled. During MSEA analysis, all normalized metabolites were reexpressed in reference to the mean value of the remission group. To identify biomarkers, univariate analysis was done using MetaboAnalyst 5.0 and significant metabolites between remission vs active group were discovered based on fold changes (FCs) and Student *t*-tests and expressed using volcano plots. For univariate analysis, a P value $<.05$ was deemed significant for pathway exploration as validated previously for pathway identification.[22] Volcano plots were constructed based on FC threshold of 1.2 and Student *t*-test threshold of $P < .05$ to identify most significant metabolite changes. For multivariate modeling, partial least squares–discriminant analysis (PLS-DA) was performed for all metabolites. PLS-DA outputs include variable important in projection (VIP), which is a weighted sum of squares of PLS. VIP scores were calculated for the top 15 leading urine and serum metabolites, with VIP score ≥ 1 signifying significant influence within the PLS-DA model. Final 3-dimensional scores plot demonstrating the PLS-DA modeling was created based on the top 15 VIP metabolites. The receiver operating characteristic analysis was built using PLS-DA metabolites by selecting the top area under the curve (AUC) variables. Model validations for both urine and serum metabolites using PLS-DA were assessed by permutations checks, whereby 40% of the data set was used as a training set and 60% of the data set was used as a testing set for modeling validation. All statistical analyses for MSEA were done using MetaboAnaylst 5.0 outputted data sets. Graphical representations including heatmaps, volcano plots, and dot plots were generated using MetaboAnalyst 5.0 and TBtools.[25]

Obstetrical Outcomes

Maternal obstetrical outcomes were collected via self-completed questionnaires or obstetrical notes at 2 weeks postpartum follow-up visits. These variables included mode of delivery (vaginal vs cesarean section), primary vs secondary cesarean section, indications, obstetrical complications (preeclampsia, placental abnormalities, or preterm premature rupture of membrane), newborn gender, and birth weight and birth length.

Enzyme-Linked Immunosorbent Assay for FCP and CRP Measurements

Maternal fecal samples were collected and stored at $-80\ ^{\circ}C$ in sterile 25-mL vials. During days of processing, stool samples were thawed and processed using Buhlmann Calex cap per manufacturer's protocols.[26] Processed samples were loaded onto 96-well plates in duplicates, and enzyme-linked immunosorbent assay was performed using FCP enzyme-linked immunosorbent assay kit based on manufacturer's protocol. CRP was quantified using V-PLEX pro-inflammatory panel human kit as described previously.[27]

Statistical Considerations

Statistical analyses were done using GraphPad Prism version 6.0 and Jamovi version 2.0. Data were represented as mean $\pm$ standard error of the mean. To detect differences between categorical variables and continuous variables, Student *t*-test was applied. Statistical analysis for multivariate regression modeling is described under metabolomics analysis. Metabolites with more than 20% missing values were omitted from the analysis.

Results

Baseline Demographics of Study Population

Table 1 details the baseline patient demographics at the time of enrollment. A total of 48 patients with IBD were included in the current study: 20 patients with CD and 28 patients with UC. Fifteen patients were recruited at preconception and 33 were recruited during trimester 1. The median age of patients was 30.5 (interquartile range [IQR], 6.5) for patients with CD and 30 (IQR, 5.5) for patients with UC. The median age of diagnosis was 22.5 (IQR, 7.8) for patients with CD and 23 (IQR, 7.0) for patients with UC. Between the 2 groups, there were comparable patient ethnicity, extent of employment, and highest education status. Social habits including smoking and alcohol use were similar across both groups (Table 1).

IBD Disease Activity at Preconception, Trimester 1, and Trimester 2

Among the 48 patients with IBD, 15 patients provided metabolite sampling (urine or serum) at preconception, 32 at trimester 1, and 15 at trimester 2. Across time points, we noted differences in IBD subtypes collected, where majority of patients sampled at trimester 2 were patients with UC, whereas those at preconception and trimester 1 were split evenly between CD and UC ($P = .02$, Table 2). For IBD disease activity monitoring, all patients received routine CRP monitoring, whereas FCP data sets were 60% complete. At preconception, 3 of 15 (20%) patients were in clinically active disease and 5 of 15 (33%) were in biochemically active disease as defined by either elevated CRP or FCP. At trimester 1, 7 of 30 (23%) patients were in clinically active disease and 10 of 32 (31%) in biochemically active disease (Table 2). Comparison across time points shows comparable medication breakdowns by trimester, where most patients were on 5-aminosalicylic acid and azathioprine at preconception, biologics and azathioprine at trimester 2, and mostly azathioprine at trimester 3 (Table 2). There was no corticosteroid use by any patient at any time point within our study.

Maternal Metabolites Differ by IBD Disease Activity and Time of Pregnancy

To compare the metabolite profiles in women with active disease vs remission, we collected a total of 66 urine samples and 58 serum samples for NMR spectroscopy. Heatmap comparison of urinary metabolites (Figure 1A) and serum metabolites (Figure 1B) revealed significant shifts by maternal disease activity as well as timing of pregnancy. Using a statistical significance of P

Table 1. Baseline Demographics

Demographic	Crohn's disease (N = 20)	Ulcerative colitis (N = 28)	*P* values
Age at enrollment[a]	30.5 (6.5)	30.0 (5.5)	.55[c]
Age at diagnosis[a]	22.5 (7.8)	23.0 (7.0)	.73[c]
Ethnicity: Caucasian	14/14	22/23	.73[b]
Employment: full-time	12/13	20/21	.72[b]
Education			.17[b]
High-school degrees	4/16	2/24	
Trades programs	7/16	8/24	
University level	5/16	14/24	
Smoking: never	8/15	12/25	.74[b]
Ex-smoker	8/15	13/25	
Alcohol			.88[b]
Active drinker	1/15	2/25	
Ex-drinker	10/15	18/25	
Never consumed	4/15	5/25	

[a]Data expressed as median (IQR).
[b]Pearson.
[c]Wilcoxon.

< .05, a total of 24 urine and 17 serum metabolites were significantly altered by biochemical disease activity across all time points (either CRP or FCP). For both urine and serum metabolites, preconception had the most disease activity-associated metabolites (Figure 1C, Tables A1–A6). Comparison across time points revealed almost no metabolites with persistent alterations throughout pregnancy (Figure 1C). Between preconception and trimester 2, urine threonine, glutamine, and phosphocreatine were persistently altered by active disease. However, no metabolites were persistently altered across all 3 time points (preconception, trimester 1, and trimester 2) in either urine or serum sampling. To understand the overall clustering by urine or serum metabolomics, we constructed principal component analysis (PCA) which showed overall poor separation in the entire metabolite profiles between active disease vs remission across time points (Figure 1D and E, PCA analysis for preconception and trimester 2 not shown). Overall, this demonstrated that active disease during pregnancy was associated with select metabolite changes, but the overall metabolite profiles were comparable across disease activity on PCA.

T1 Active Disease-Associated Metabolites Enrich to Amino Acid and Lipid Metabolism

To understand the importance of the earliest metabolite changes during pregnancy, we carried out MSEA using trimester 1 metabolome. Using Reactome pathway topology analysis,[28] T1 active disease-associated urine metabolites were significantly enriched in "arginine and proline metabolism,"

Table 2. IBD Characteristics by Timing of Metabolomics Analysis

IBD characteristics	Preconception (N = 15)	Trimester 1 (N = 32)	Trimester 2 (N = 15)	*P* values
IBD subtype: UC	8/15	16/32	13/15	.02[b]
Time of collection[a] (weeks GA)	-	7.9 (6.2–10.9)	15.6 (14.9–17.2)	<.01[c]
Medications				
Biologics use	6/14	15/29	4/13	.45[b]
5-ASA use	7/14	6/29	5/13	.13[b]
Azathioprine use	7/14	14/30	2/13	.10[b]
Corticosteroids use	0/14	0/32	0/13	-
Disease activity				
Clinical: pMAYO ≥2 or HBI ≥5	3/15	7/30	3/15	.98[b]
Biochemical: CRP ≥10 mg/L	1/15	4/28	5/15	.08[b]
Biochemical: FCP ≥250mg/g	4/10	6/16	3/15	.89[b]
Active disease by CRP/FCP	5/15	10/32	6/15	.73[b]

5-ASA, 5-aminosalicylic acid; GA, gestational age.
[a]Data expressed as median weeks GA (CI).
[b]Pearson.
[c]Wilcoxon.

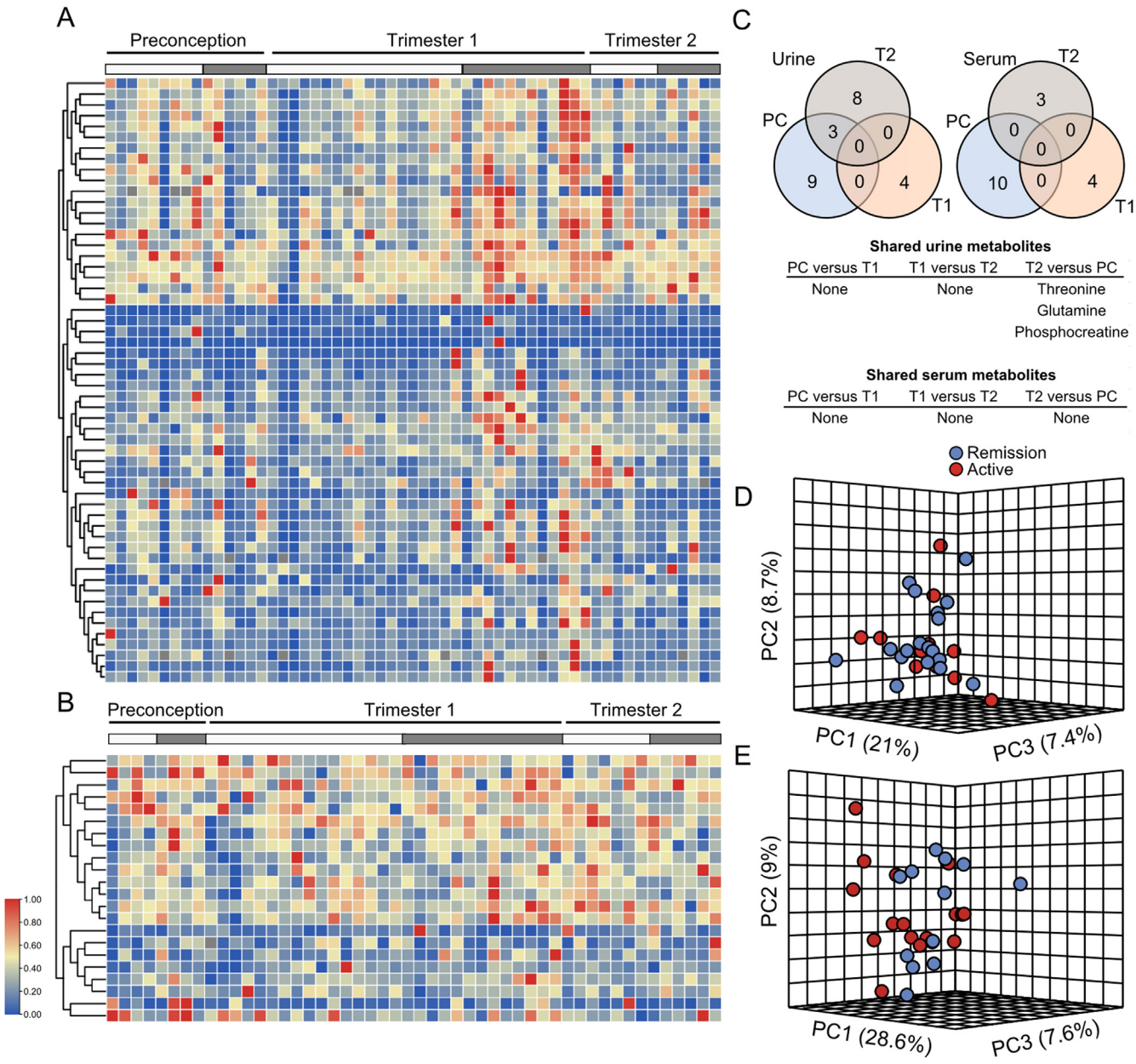

Figure 1. Metabolomic profiles of pregnant women with IBD in active disease vs remission. (A) Heatmap representation displaying the significantly altered urine and (B) serum metabolite changes across preconception, trimester 1, and trimester 2 (N = 15–32 per time point, $P < .05$). (C) Venn diagram illustrating the breakdown of significant metabolites unique or shared across time points. (D) Principal component analysis displaying the separation of urine and (E) serum metabolomic profiles between trimester 1 active disease vs remission.

"fatty acid biosynthesis and β-oxidation," and "ketone body metabolism" (Figure 2A), whereas the T1 active disease-associated serum metabolites were enriched in "glycolysis," "fatty acid biosynthesis," and "lactose synthesis and degradation" ($P < .05$, Figure 2B). Using enrichments by compound classes, we noted that the majority of T1 active disease-associated metabolites were amino acid derivatives ($P < .05$), fatty acid derivatives, or phospholipids (Figure 2C). To explore the directionality of metabolite changes, metabolites were normalized by FC and analyzed using volcano plots. At T1, the 2 most downregulated urine metabolites were succinic acid and 3-hydroxy-2-methylbutyric acid (Figure 2D), whereas the most upregulated metabolites were C10:2, trimethylamine N-oxide, and 4-hydroxymandelic acid. On the other hand, the only significant downregulated serum metabolite was acetic acid, and the top upregulated metabolites were glucose, trimethylamine N-oxide, and C6 (Figure 2E). Taken together, these results show that T1 disease activity-associated metabolites were enriched in amino acid metabolism and differ by samples.

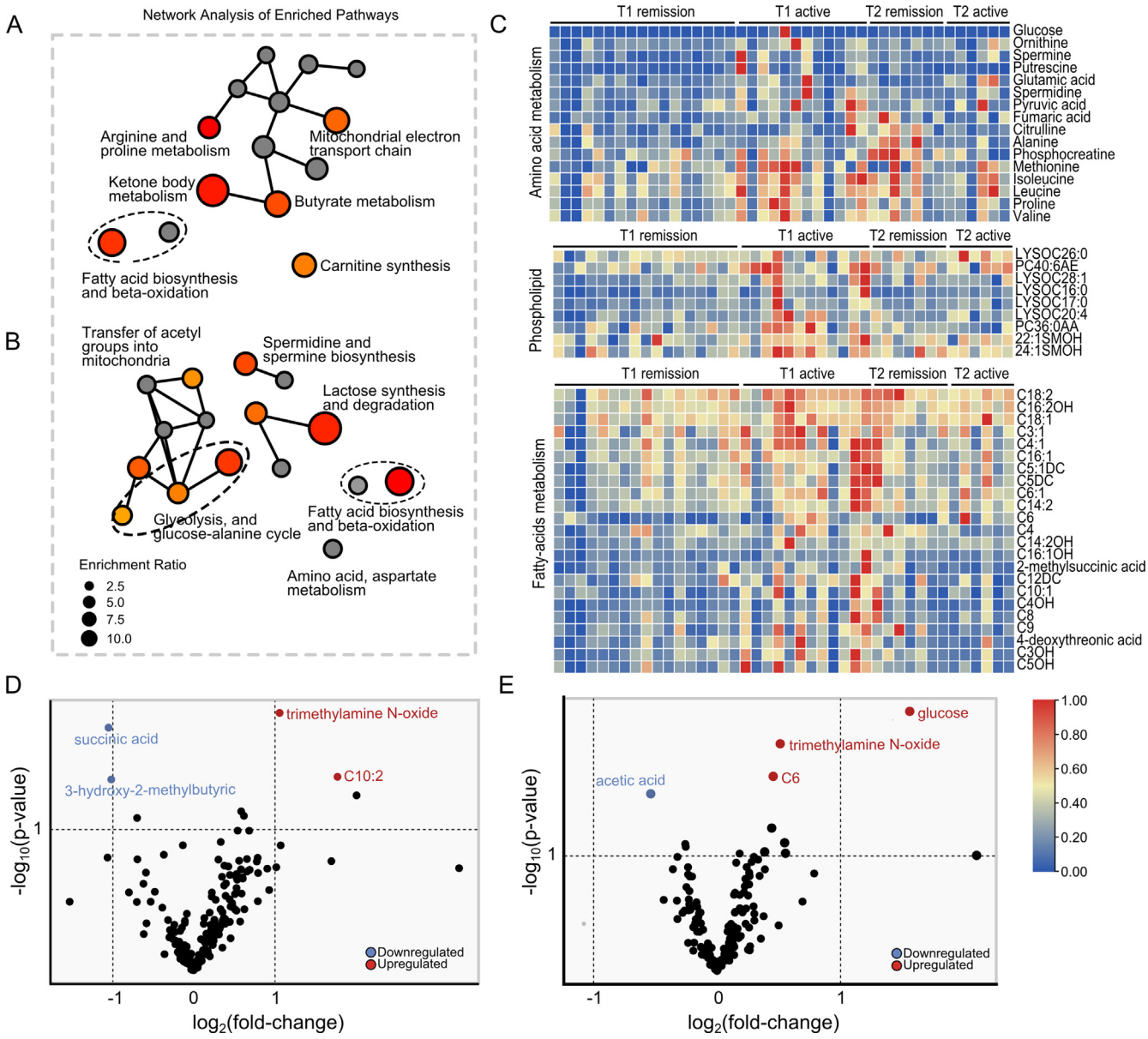

Figure 2. Biological enrichments of metabolomics by Reactome pathways. (A) Reactome pathway topology analyses for differentially altered urine and (B) serum metabolites (false discovery rate < 0.001) pathways were colored; grey denotes nonsignificant enrichments; size of node denotes enrichment ratio. (C) Heatmap showing changes in urine metabolites classified by class of compounds. (D) Volcano plots demonstrating all urine and (E) serum metabolites significantly altered at trimester 1. Red denotes upregulated metabolites and blue denotes downregulated metabolites during active disease at trimester 1 ($P < .05$, FC > 1.2). Dotted horizontal lines demonstrate significance threshold, and vertical lines denote FC threshold of 1.2. PC, preconception; T1, trimester 1; T2, trimester 2.

Separating Active vs Remission at Trimester 1 Using PLS-DA Multivariate Modeling

To identify the metabolites most important to discriminating active disease and remission, we performed PLS-DA using T1 urine and serum metabolites. The leading metabolites in discriminating women with active disease from remission were ranked using VIP scores (Figure 3A), with normalized changes shown (Figure 3B). To test the discriminatory efficiency of the PLS-DA modeling, we constructed PLS-DA score plots for urine and serum metabolites, which showed clear separation between active disease and remission at T1 (Figure 3C and D). Repeated permutation testing demonstrates that observed separation in both metabolite samples was not due to chance (data not shown). Specifically, the 5 urine metabolites with leading VIP scores were 3-hydroxy-2-methylbutyric acid, trimethylamine-N-oxide (TMAO), succinic acid, C10:2, and 4-hydroxymandelic acid (Figure 3E). In serum samples, metabolites with highest VIP scores were glucose, TMAO, C6, acetic acid, and uric acid (Figure 3E, full normalized metabolite changes in Supplemental Materials). To explore the

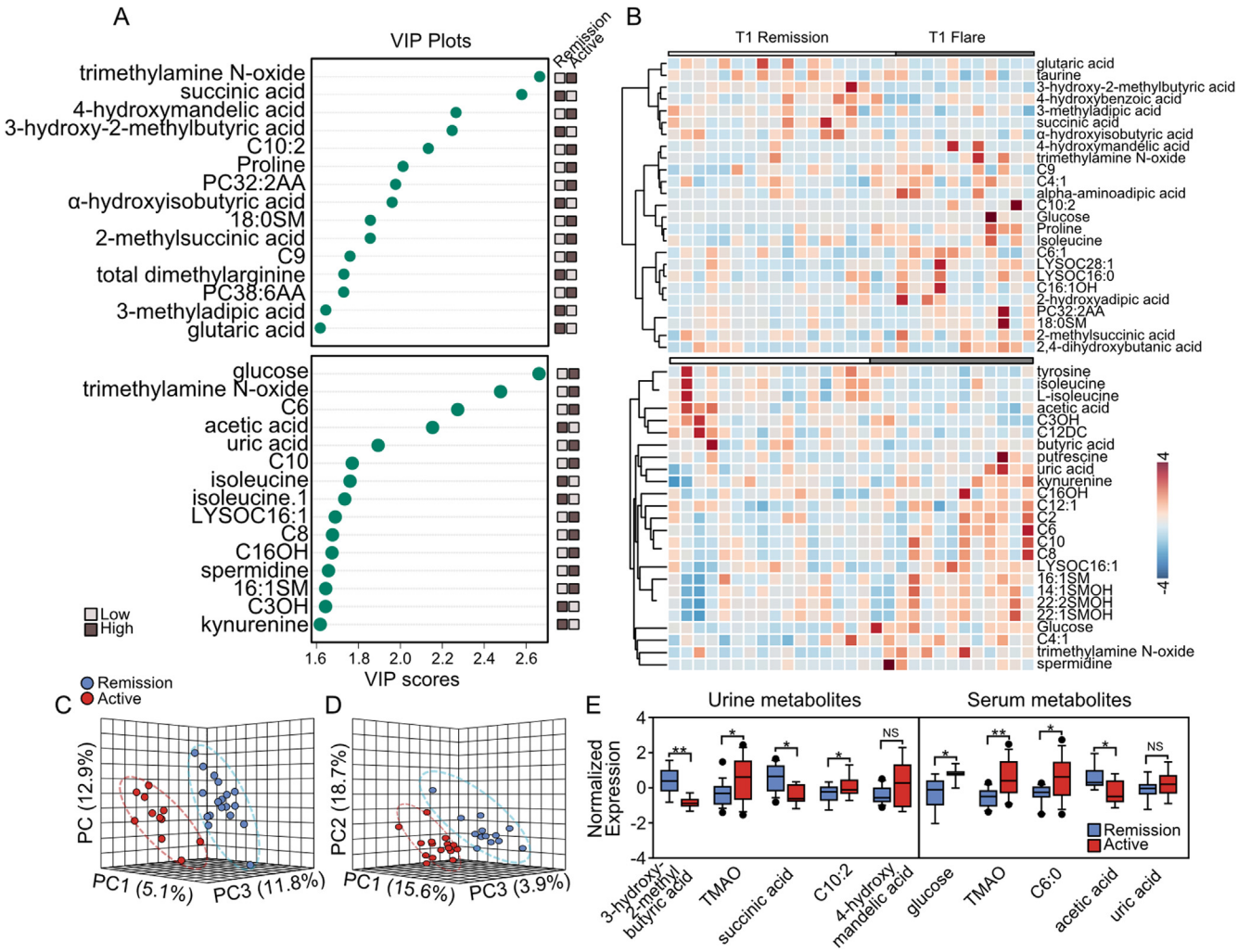

Figure 3. PDS-LA modeling to discriminate active disease vs remission at trimester 1. (A) VIP score plots demonstrating the top 15 T1 disease activity-associated metabolites (urine on top, serum on bottom, N = 32). (B) Heatmap representation of the leading VIP metabolites for urine (top) and serum (bottom) metabolomic profiles. (C) Principal component analysis showing patient clustering based on top 15 urine or (D) serum metabolites. (E) Boxplots demonstrating the top 5 leading VIP urine and serum metabolites associated with active disease at trimester 1. * denotes $P < .05$, ** denotes $P < .005$. PC on axes labels defined as principal component.

possibility that the observed changes could be due to dietary supplements, we tracked the use of supplements throughout pregnancy, which showed comparable rates in the exogenous use of multivitamins, iron, folate, cod liver oil, omega-3, calcium, and vitamin D supplements between women with T1 remission vs active disease (Table A8). Additionally, while there was a trend for decreased ferritin levels in women with active disease, there were no differences in serum vitamin B12 or ferritin levels at T1—2 markers of malnutrition (Table A8). Taken together, this analysis demonstrates that a multivariate model based on few select important metabolites may discriminate active disease vs remission at the time of sampling.

Multivariable Regression Using 3 Metabolites Discriminates Active Disease From Remission

To test the clinical correlation for the identified leading metabolites, we developed stepwise logistic regression models using top 3 metabolites selected based on VIP scores and AUCs. In urine metabolites, the leading metabolites incorporated were 3-hydroxy-2-methylbutyric acid, TMAO, and succinic acid (Figure 4A). In serum metabolites, the leading metabolites were TMAO, glucose, and acetic acid (Figure 4B). Both models were developed from a training data set using 40% of samples and validated using the test group (60% of samples) using 1000 permutation testing (AUC = 0.719, $P = .09$ for urine metabolites; AUC = 0.918, $P < .001$ for serum metabolites; Figure 4C and D). However, the diagnostic modeling prediction showed poor prediction accuracy using urine metabolites at T1, whereas serum metabolites showed sustained significant discrimination after 1000 permutation testing ($P = .00434$, Figure 4C and D). In addition, the IBD cohort at T1 was separated using PCA testing by top 3 serum metabolites (Figure 4E).

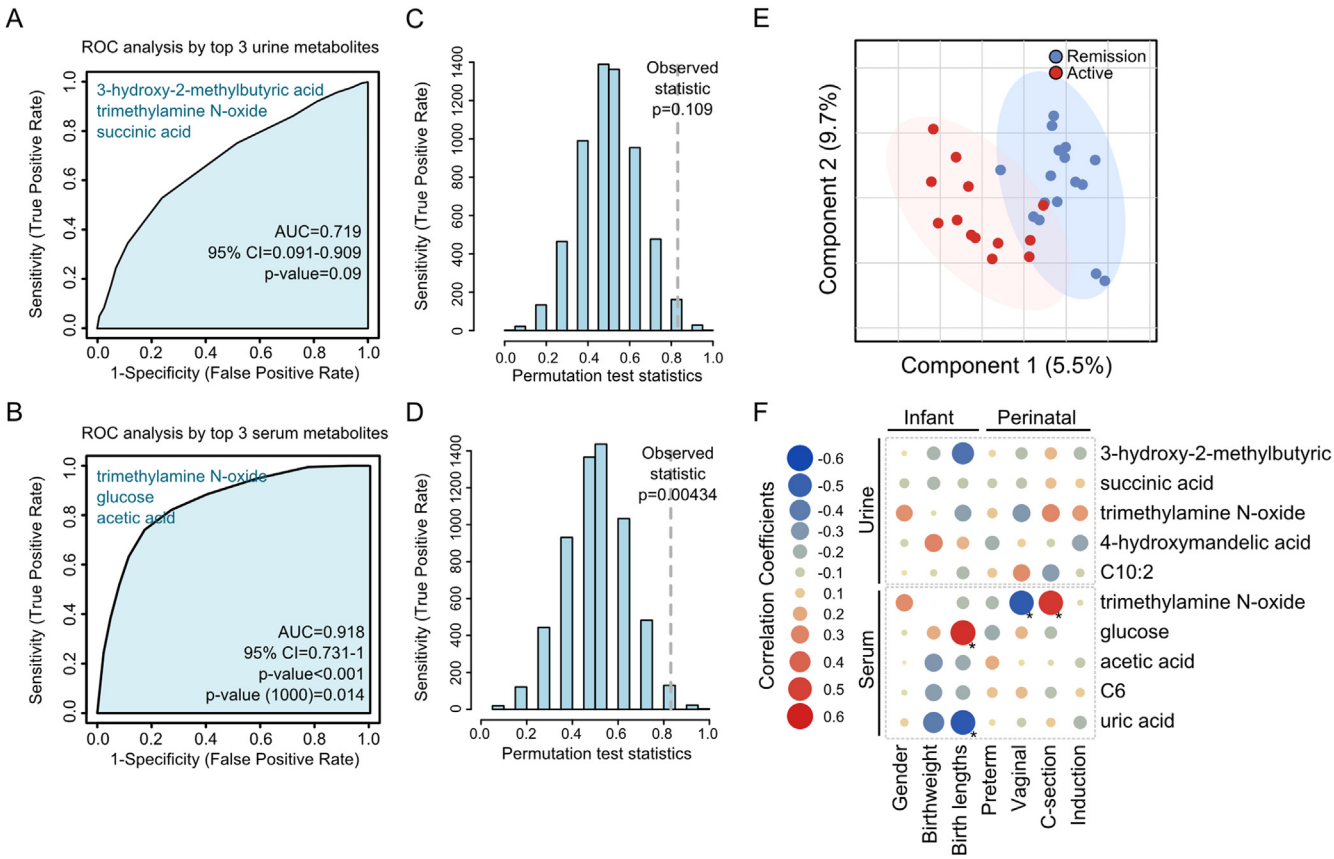

Figure 4. Metabolomic prediction using urine and serum metabolites for disease activity. (A) Receiver operating characteristic (ROC) analysis using 3 leading metabolites based on highest ranked AUC for urine or (B) serum metabolites (N = 28 for urine metabolites, N = 32 for serum). (C and D) Modeling prediction using 1000 permutations to test urine and (D) serum metabolite PDS-LA modeling. (E) PCA analysis demonstrating separation in patients based on 3 leading serum metabolites. (F) Dot plots demonstrating Pearson correlations between clinical outcomes and leading VIP metabolites (* denotes Pearson correlation coefficients with $P < .05$).

To further interrogate the clinical relevance of the identified metabolites, we correlated the leading metabolites with neonatal and obstetrical outcomes at delivery (Figure 4F, detailed obstetrical outcomes of mothers with T1 metabolites shown in Table A7). Pearson correlation coefficients demonstrated poor correlation between urine metabolites and clinical outcomes at delivery (Figure 4F). On the other hand, serum glucose and uric acid correlated with infant birth length, and serum TMAO correlated with mode of delivery (Figure 4F). Overall, this demonstrates that a multivariable modeling involving serum glucose, acetic acid, and TMAO discriminates T1 active disease from remission and has clinical correlation with neonatal and obstetrical outcomes.

Combination of Leading Urine and Serum Metabolites Predict Future Disease Activity in Pregnancy

Finally, to test the ability of metabolites to predict disease activity across time points, we combined all urine and serum metabolites and performed PLS-DA against the disease activity at *subsequent time points*. Specifically, preconception metabolites grouped based on T1 disease activity (Figure 5A), T1 metabolites grouped based on T2 disease activity (Figure 5B), and T2 metabolites grouped based on T3 disease activity (Figure 5C). Across all 3 prediction models, there was a complete separation of disease status based on 3 leading VIP metabolites. Preconception levels of urinary 2,4-dihydroxybutanoic acid, Sumiki's acid, and 2,5-furandicarboxylic acid completely predicted T1 disease status of the women in our cohort (AUC = 1; 95% CI, 0.95–1), although there was insufficient training sample set to statistically test the model (Figure 5D). Furthermore, the combination of serum 18:0SM, LYSOC16:1, and urinary 4-hydroxyhippuric acid predicted T2 disease status (AUC = 0.942; 95% CI, 0.8–1) with training set validation ($P = .006$, Figure 5E). At trimester 2, the leading urinary metabolites were ethylmalonic acid, 2-oxadipic acid, and 3-methylglutaric acid (AUC = 0.72; 95% CI, 0.2–1), but the model was not statistically significant in training set validation ($P = .15$, Figure 5F). Across all 3 prediction models, the 3 leading metabolites correlated with future disease activity (Figure 5G) and demonstrated high predictive values in all 3 training data sets (Figure 5H, summary model in Figure 5I).

Overall, this demonstrates that metabolite changes correlate and predict disease activity at future time points.

Discussion

Achieving and maintaining remission in preconception and pregnancy is the current recommendation for managing IBD in pregnant women.[1,29] This is challenging using conventional approaches given potential risk of invasive tests to the developing fetus and physiologic variation in serum biomarkers throughout pregnancy.[30] Our study investigated the applicability of metabolomics to discriminate patients with active disease vs those in remission during pregnancy. We demonstrate that (1) active disease in pregnancy is correlated with specific metabolite changes which vary by trimester and biological sampling (urine vs serum); (2) multivariable regression modeling using serum TMAO, glucose, and acetic acid discriminate active disease from remission; (3) serum metabolites individually correlate with infant and obstetrical outcomes at delivery; and (4) select metabolites may predict future active disease later in pregnancy. To our knowledge, this is the first study to date to comprehensively interrogate the metabolome changes of women with IBD during pregnancy.

The application of metabolomics to disease diagnosis has been well studied given their advantage in noninvasive testing and biological correlations.[16] Specifically, metabolomic differences have been extensively explored in other disease settings such as colorectal cancer,[31] renal cell carcinoma,[18] and skin cancer.[32] However, the application of metabolomics in IBD remains unclear. Previously, in comparisons between IBD vs healthy controls, IBD was associated with decreased levels of fecal short-chain fatty acids and polyamines[33]; higher urine succinate,[19] citrate, and hippurate[20]; and altered levels of serum lipids and amino acid derivatives.[34] However, few reports have examined the metabolite changes associated with disease activity, and particularly during pregnancy. Previously, De Preter et al[35] showed hexanoate levels were inversely correlated with disease activity in patients with CD. However, no studies so far have interrogated these changes in pregnant women with IBD, and only one study to date has examined metabolite changes in breastmilk.[27]

The metabolite profiles of serum samples allowed clear separation of women with active disease from those with remission. In fact, a 3-metabolite prediction model using TMAO, glucose, and acetic acid allowed for robust prediction of disease activity. TMAO is a microbiota-derived metabolite generated with ingestion of dietary choline or choline-containing compounds.[36] TMAO metabolism involves the breakdown of choline into trimethylamine—a TMAO precursor.[36] In preclinical models, TMAO activates host pro-inflammatory pathways including mitogen-activated protein kinase[37] and nuclear factor-κB signaling[38] and increases cytokine expression and NLR family pyrin domain containing 3 inflammasomes.[39] In select in vivo models, ingestion of TMAO causes glucose intolerance[40] and is linked to microbial dysbiosis in various disease settings including colorectal cancer, cardiovascular disease, and chronic kidney disease.[41] In pregnancy, elevated TMAO is associated with impaired glucose tolerance and pre-eclampsia.[42,43] Our finding that TMAO as the only metabolite consistently upregulated in both urine and serum samples of mothers with active trimester 1 disease fits the existing literature. One explanation could be that elevated TMAO is the result of gut microbiota changes at trimester 1. This is supported by the fact that both acetic acid (decreased) and glucose (increased) were altered in T1 active mothers. Acetic acid is a microbiota-derived short-chain fatty acid and its decrease was also previously shown in patients with CD.[44] The concurrent changes in TMAO and acetic acid may reflect a change in the maternal microbiome at T1, thereby correlating with overall host inflammation and elevated glucose. Further studies incorporating microbiome analysis would be warranted to validate this hypothesis.

Besides TMAO, the metabolite changes varied based on biological samples (serum vs urine) and by timing of pregnancy. This is congruent with existing reports, whereby pregnancy-related metabolome fluctuates by week of gestation and highest in early trimester,[45] likely due to the significant organogenesis that occurs during trimester 1. Our metabolome data showing only few metabolite alterations by trimester 2 also reflect this variation. In addition, the difference between urine vs serum metabolites in discriminating disease activity is intriguing. While we noted more urine metabolite changes, multivariable modeling using T1 urine metabolites was not as reliable as serum metabolites *at the time of collection*. In contrast, the combination of TMAO, acetic acid, and glucose was more reliable and robust in permutation testing. This difference could be due to physiologic variation and timing of sample collections. For instance, urine metabolites are known to be more volatile, incur larger physiologic variations compared to serum metabolites,[46] and interestingly, only later trimesters urine metabolites appear to predict clinically relevant outcomes.[47,48] Our current study did not complete metabolite profiling up to trimester 3, but it would be intriguing to see if late trimester 3 urinary metabolites have any predictive values in neonatal outcomes in future studies.

Importantly, besides discrimination of disease activity at trimester 1, we also show that early metabolite changes predict and correlate with clinical outcomes at subsequent trimesters. Specifically, we generated a reliable multivariable model using T1 metabolites (serum 18:0SM, serum LYSOC16:1, and urine 4-hydroxyhippuric acid) to predict T2 disease activity. Similarly, preconception levels of 3 urine metabolites predicted T1 disease activity within our cohort, although this model could not be validated further given lack of sample size for training set. Moreover, we also show T1 serum metabolites correlated well with mode of delivery and infant growth data such as birth length. Based on the current literature, these are by far the earliest time points

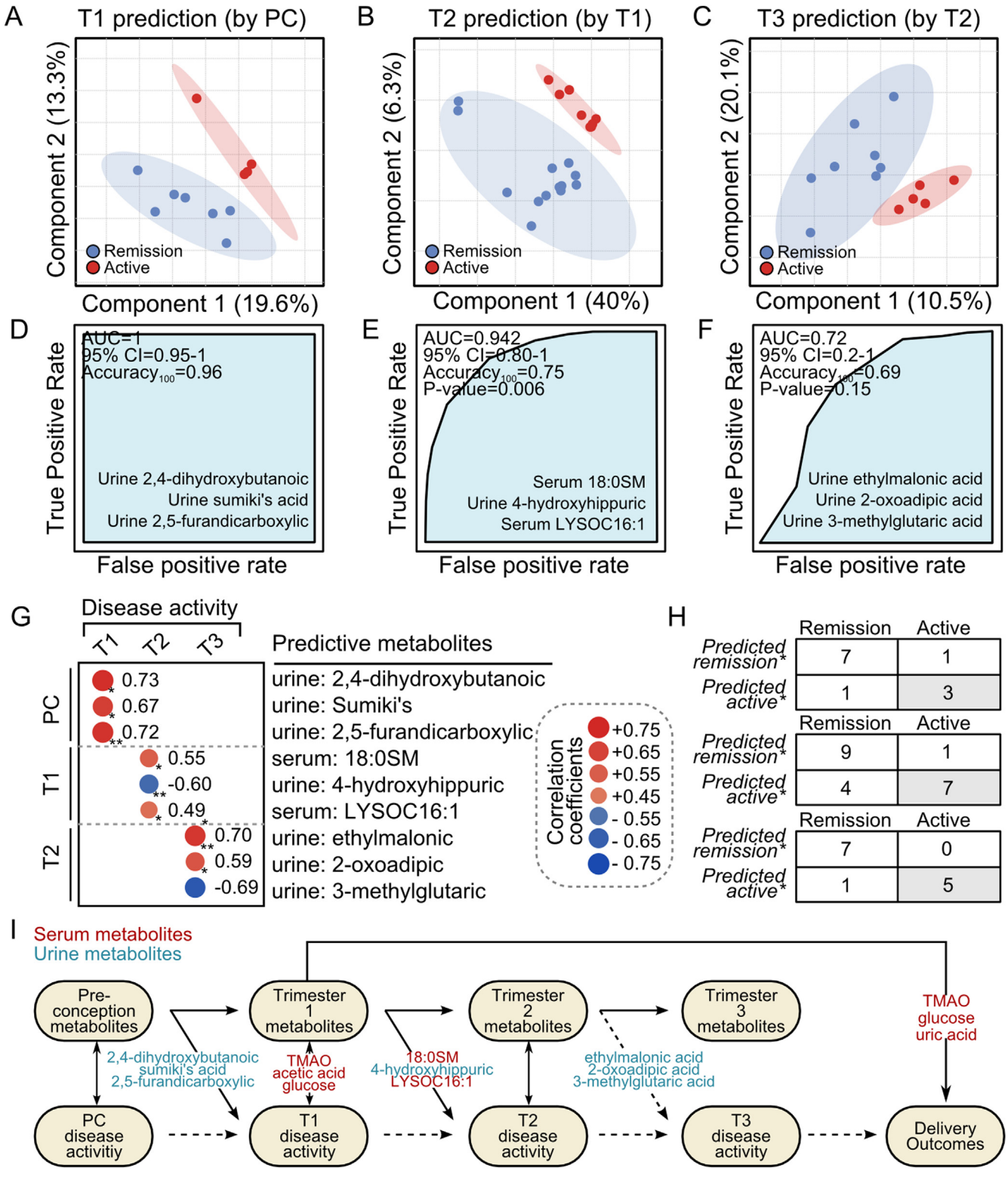

Figure 5. Metabolomic prediction for future disease activity. (A–C) PCA analyses at each time point predicted by the leading urine or serum metabolites of prior time point. (D–F) Receiver operating characteristic (ROC) analyses using 3 urine or serum metabolites of preceding time point ranked based on highest AUCs. Permutation testing done using leading metabolites by 40% training set and 60% testing set. (G) Pearson correlation coefficients between predictive metabolites and disease activity at subsequent time point ($P < .05$, numbers within plot denotes Pearson correlation coefficients). (H) Confusion matrices using validated modeling (top is T1 prediction by preconception metabolites, followed by T2 prediction by T1 metabolites, and bottom is T3 prediction by T2 metabolites). (I) Overall schematic showing metabolomics predictions. * denotes $P < .05$, ** denotes $P < .005$. PC, preconception; T1, trimester 1; T2, trimester 2.

by which metabolite outcome correlations are drawn to this specific population. This finding also correlates with our recent reports, where we showed that trimester 1 disease activity was correlated with worse neonatal and obstetrical outcomes at the time of delivery.[4] Recently, we also showed that active disease during trimester 1 could reflect growth differences in infants up to 6 months of age. These results fit with our previous findings and support the theory where trimester 1 active disease perturbs the inflammatory environment and leads to higher risk of complications during pregnancy. While our study only followed neonatal outcomes, larger studies with extended postneonatal follow-up will be warranted. For instance, Kim et al[49] recently showed that growth difference in offspring between patients with IBD and healthy controls may persist up to 6 years of age.

Although our study discovered discriminant metabolite markers with clinical correlations to delivery outcomes, there are several limitations that will require further clarification. Foremost, our cohort study was completed in 2014–2017, and since then, novel studies have shown that specific dietary patterns, such as vegetarian vs nonvegetarian,[50] amount of complex carbohydrate intake,[22] can influence urine and serum metabolite changes as recently reviewed elsewhere.[51] For instance, recent study shows that specific dietary patterns such as Mediterranean diets are associated with shifts in gut microbiome to affect the abundance of fibre-degrading commensals.[52] Similarly, the amount of fiber intake is also associated with significant shifts in fecal metabolites, short-chain fatty acids, and changes in the serum metabolome in healthy adults.[53] Within our study, even though we attempted to assess nutritional status by assessing the patients' use of exogenous supplements, we cannot fully rule out potential interactions from core dietary patterns given the newly published data on host metabolomic profiles. Likewise, calories consumed were also not measured during our study, and therefore, it may be possible that our identified metabolites could be due to changes in diet between remission and flare-up. Further studies to stratify patients based on more current dietary metrics such as "food frequency questionnaires" to characterize these patterns are currently underway.[51] Secondly, while our cohort size is comparable to previous metabolomic studies in IBD, our study population lacks heterogeneity. For instance, despite the study being conducted at a tertiary center, our cohort demographics are limited to women who were mostly Caucasian, married, and had postsecondary education. Similarly, the IBD characteristics of our cohort were also milder in terms of activity with no patients requiring corticosteroids during pregnancy. Therefore, further studies using larger, more diverse cohorts are warranted to substantiate our pilot study findings. Furthermore, there were notable losses to follow-up from missed or canceled appointments particularly at trimester 3. Among all 48 patients enrolled, the losses to follow-up were about 15% and 20%–40% missing data collection. Although we excluded variables with more 40% missing data, the losses to follow-up prevented the statistical modeling at later trimesters. Lastly, as it was not feasible within our study to perform endoscopy to assess disease activity during pregnancy, the physiologic variation in FCP and CRP may undermine the validity of maternal disease activity. Further studies to correlate the identified metabolites against endoscopic evaluation are therefore warranted to validate these findings.

Nevertheless, to the best of our knowledge, this is the first pilot study to date to comprehensively follow metabolic derangements in pregnant women with IBD from preconception to trimester 2 of pregnancy. Despite our limitations in monitoring dietary patterns and lack of sample size, our work is hypothesis-generating as it suggests the discriminatory potential of serum metabolites in separating active disease from remission, as well as correlations between select metabolites and clinical outcomes at delivery, and potentially a predictive utility in monitoring disease activity. Further understanding of the specific metabolome changes could offer further insights into pathophysiology of active disease during pregnancy and identify important biomarkers for diagnostic and monitoring of disease, as well as the promise of rational novel therapies that could improve pregnancy outcomes.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2022.07.008.

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Received April 1, 2022. Accepted July 11, 2022.

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Acknowledgments:
We thank all the referring gastroenterologists, nurses, and the participating women with IBD who contributed to this research study. We also thank all the participating summer students, research student Rowan Lumb, research coordinator Reed Sutton and members of TMIC, Dr Wishart and his team for processing the metabolomic samples. Lastly, we express our deepest gratitude and remembrance of the late Dr Richard Fedorak (1955–2018) who was instrumental in the initial conception and design of this research project.

Authors' Contributions:
Dr Vivian Huang takes responsibility for the integrity of the work in its entirety from inception to the final published manuscript. Richard Y. Wu was involved in study data acquisition, data analysis, interpretation of data, drafting of manuscript, and revising critically the manuscript. Parul Tandon and Joyce S. Oh were responsible for acquisition of data, interpretation of data, and final approval of the version submitted. Lindsy Ambrosio, Naomi Hotte, and Binal Shah-Gandhi were responsible for data acquisition, data analysis, and revising critically for important intellectual content. Karen Madsen, Levinus A. Dieleman, Shokrollah Elahi, and Karen I. Kroeker were responsible for conception and design of the study, analyses and interpretation of the data, and revising critically for important intellectual contents. All authors have approved the final version of the article and the final full authorship list. No writing assistance was required during preparation of the manuscript.

Conflicts of Interest:
The authors disclose no conflicts.

Funding:
The project is supported by local supports from CEGIIR, University of Alberta Departments of Medicine and Gastroenterology, and Mount Sinai Hospital, Department of Medicine.

Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:
The data that support the findings of this study are not openly available due to reasons of patient confidentiality and are available from the corresponding author and affiliated institutions upon reasonable request.