## Title

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## Journal

Clinical Cancer Research, 30(14)

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## Publication Date

2024-07-15

## DOI

10.1158/1078-0432.CCR-23-2934

# Cervicovaginal Metabolome and Tumor Characteristics for Endometrial Cancer Detection and Risk Stratification

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## ABSTRACT

**Purpose:** Endometrial cancer is highly prevalent and lacking noninvasive diagnostic techniques. Diagnosis depends on histological investigation of biopsy samples. Serum biomarkers for endometrial cancer have lacked sensitivity and specificity. The objective of this study was to investigate the cervicovaginal environment to improve the understanding of metabolic reprogramming related to endometrial cancer and identify potential biomarker candidates for noninvasive diagnostic and prognostic tests.

**Experimental Design:** Cervicovaginal lavages were collected from 192 participants with endometrial cancer ($n = 66$) and non-malignant conditions ($n = 108$), and global untargeted metabolomics was performed. Using the metabolite data ($n = 920$), we completed a multivariate biomarker discovery analysis.

**Results:** We analyzed grade 1/2 endometrioid carcinoma ($n = 53$) and other endometrial cancer subtypes ($n = 13$) to identify shared and unique metabolic signatures between the subtypes. When compared to non-malignant conditions, downregulation of proline ($P < 0.0001$), tryptophan ($P < 0.0001$), and glutamate ($P < 0.0001$) was found among both endometrial cancer groups, relating to key hallmarks of cancer including immune suppression and redox balance. Upregulation ($q < 0.05$) of sphingolipids, fatty acids, and glycerophospholipids was observed in endometrial cancer in a type-specific manner. Furthermore, cervicovaginal metabolites related to tumor characteristics, including tumor size and myometrial invasion.

**Conclusions:** Our findings provide insights into understanding the endometrial cancer metabolic landscape and improvement in diagnosis. The metabolic dysregulation described in this article linked specific metabolites and pathophysiological mechanisms including cellular proliferation, energy supply, and invasion of neighboring tissues. Furthermore, cervicovaginal metabolite levels related to tumor characteristics, which are used for risk stratification. Overall, development of noninvasive diagnostics can improve both the acceptability and accessibility of diagnosis.

## Introduction

Endometrial cancer is a cancer of the lining of the uterus and is the most prevalent gynecologic malignancy in high-income countries (1), with an annual global estimated incidence of 417,000 new cases and 97,000 deaths (2). Risk factors of endometrial cancer include increased body mass index (BMI), diabetes, older age, early menopause, hypertension, family history (Lynch syndrome), and polycystic ovary syndrome (3–5). Atypical hyperplasia is also a major risk factor for the development of endometrial cancer when left untreated, being an endometrial precancerous condition (6).

Current diagnosis of endometrial cancer relies on histopathological investigation of biopsy samples. Endometrial specimens are typically collected at a physician's office using biopsy pipelles with or without hysteroscopy or dilation and curettage (D&C; ref. 7). However, certain populations of women (e.g., morbidly obese, mentally disabled, sexually traumatized) often require the operating room setting for biopsy sample collection. Imaging techniques, specifically transvaginal ultrasound, computed tomography (CT) scans, and magnetic resonance imaging (MRI), are used preoperatively in endometrial cancer management. Through assessment of factors including myometrial invasion, lymphovascular invasion, and tumor size, these techniques are more reliable for preoperative characterization of endometrial cancer, as well as for continually monitoring and management of endometrial cancer; yet, they lack sensitivity and specificity for diagnostic value in endometrial cancer (8). Regarding biomarkers, serum levels of two proteins, human epididymis protein 4 (HE4) and cancer antigen 125, have been shown to be altered in endometrial cancer (7, 9–11), but these markers also lack sensitivity and specificity and are more indicative for prognosis than diagnostic purposes (12, 13).

Endometrial cancer was previously grouped into two major categories: type I [consisting of grade 1 and 2 endometrioid carcinoma (EEC)] and type II (composed of higher grade EECs and other non-endometrioid subtypes). Now, due to endometrial cancers' heterogeneity different subtypes have been proposed based on histology and genetic information, such as mutations in p53, mismatch repair (MMR) proteins, and DNA polymerase epsilon catalytic subunit

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Clin Cancer Res 2024;30:3073–87

**doi:** 10.1158/1078-0432.CCR-23-2934

## Translational Relevance

Endometrial cancer is the fourth most common cancer among women, yet it lacks noninvasive diagnostic tools. In this study, we combined the use of cervicovaginal lavage sampling and a global untargeted metabolomic approach to identify potential biomarkers for endometrial cancer. Strikingly, our ability to link cervicovaginal metabolite levels with tumor characteristics including tumor size, histological grade, and myometrial invasion may become a key clinical translation and may be used for risk stratification. Our findings may improve diagnosis, as well as provide better understanding of metabolic reprogramming related to endometrial cancer. The local metabolomic landscape of endometrial cancer described in this article provides insights into the metabolic dysregulation present in the disease, linking to both disease progression and severity; some identified metabolites, such as sphingolipids, could be targeted for therapies in the future. Our work takes us closer to development of noninvasive tests for endometrial cancer, improving testing accessibility and comfort for patients.

gene (POLE; ref. 14). Grade 1/2 EEC is more common, is estrogen driven, and has a better prognosis (1); grade 3 EEC and other endometrial cancer subtypes are less common, are not estrogen driven, and have poorer prognosis (7).

Several factors determine the prognosis of endometrial cancer, including age, histological grade, tumor size, presence of lymphovascular or myometrial invasion, and MMR protein status (15–17). Regardless of prognosis, the gold standard for endometrial cancer treatment remains total hysterectomy, removal of ovaries and tubes and either sentinel or complete pelvic lymphadenectomy (4), however, this procedure can be undesirable, especially for younger women wanting to preserve fertility and/or prevent early menopause. Increased pathophysiological understanding and discovery of novel diagnostic and prognostic biomarkers are crucial for better risk stratification and improved treatment options for patients with endometrial cancer.

Cervicovaginal lavages (CVLs) are a washing of the vagina and cervix, which can be analyzed for biomarker discovery. Our previous research has shown CVLs potentially mirror the female reproductive tract environment and, when combined with a global metabolomics approach, can provide pathophysiological insights into various gynecologic conditions, including lower genital tract infections [e.g., human papillomavirus (HPV) infection] and cervical neoplasm as well as, more recently, upper reproductive tract conditions, including adenomyosis (18–23).

Metabolomic investigation into endometrial cancer based on noninvasive sampling is lacking; a study of this magnitude using CVLs has not previously been conducted, with only one limited study [54 participants with endometrial cancer ($n = 21$) and controls ($n = 33$)] conducted previously (24). Current literature shows a metabolomics approach has been utilized for biomarker discovery broadly in blood and tissue samples but not in other biological fluids, such as urine or CVLs (25–28). Previous analyses of serum, urine, and intrauterine brushing samples from patients with endometrial cancer revealed alterations in amino acid (including glutathione, tyrosine, alanine, aspartate, and glutamate metabolism) and nucleotide (purine and pyrimidine) pathways and potential for a metabolomics approach to be used for screening/diagnosis of endometrial cancer, particularly in symptomatic women (25, 28).

In this study, we utilized an untargeted global metabolomics platform in combination with cervicovaginal sampling from a cohort of well-characterized patients undergoing hysterectomy ($n = 192$) to identify metabolomic profiles in women with endometrial cancer. This foundational knowledge is essential for advancing pathophysiological understanding of the disease, as well as improving detection and risk stratification based on tumor progression and characteristics.

## Materials and Methods

### Ethics approval and consent to participate

This study was approved by the Institutional Review Board at the University of Arizona (IRB no. 1708726047). All participants provided informed written consent, and the study was performed in accordance with Declaration of Helsinki and federal guidelines.

### Study participants

Participants were recruited at three clinical sites in the Phoenix metropolitan area: Banner University Medical Center–Phoenix, Valleywise Health Medical Center, and Dignity Health Chandler Regional Medical Center between June 2018 and February 2020. A total of 192 women undergoing hysterectomy for benign or malignant indications were enrolled. Histopathology results from biopsy samples collected from surgery were used to stratify participants into four disease groups: benign conditions ($n = 108$; including adenomyosis, endometriosis, fibroids, and other benign implications as singular or comorbidities), endometrial hyperplasia ($n = 18$), grade 1 or 2 endometrioid carcinoma (grade 1/2 EEC; $n = 53$), and other endometrial cancer subtypes ($n = 13$; including grade 3 EEC, serous carcinoma and other histological subtypes). A breakdown of the diagnosis of hyperplasia and endometrial cancer subtypes can be found in Supplementary Table S1. We included women of any race or ethnicity and age 18 years or older. Patients were excluded based on factors such as menstruation, infectious diseases, lifestyle choices, and other conditions; detailed exclusion criteria are available in Supplementary Table S2. The exclusion criteria were verified by physician's pelvic exam, medical records, and/or self-reported data. Demographic, socioeconomic, and medical history data were collected from surveys and/or medical records.

### Sample collection and processing

CVL samples were collected by a surgeon in the operating room during a standard-of-care hysterectomy procedure. Samples were obtained after induction of anesthesia and prior to vaginal preparation with antiseptic solution. CVLs were collected using a nonlubricated speculum and 10 mL of sterile 0.9% saline solution (Teknova). Samples were immediately placed on ice and frozen at −80°C within an hour. Prior to downstream analyses, the samples were thawed on ice; centrifuged (700 × $g$ for 10 minutes at 4°C); aliquoted to prevent multiple freeze-thaw cycles; and stored at −80°C.

### Quantification of soluble metabolites

Soluble metabolites in CVL samples were quantified using a global metabolomics platform at Metabolon, Inc. as previously described (20). Briefly, the Metabolon's platform utilized a Waters ACQUITY ultra-performance liquid chromatography and a Thermo

Scientific Q-exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source and Orbitrap mass analyzer operated at 35,000 mass resolution. Compounds were identified using Metabolon's library of purified standards. Peaks were quantified using area under the curve (AUC) for relative intensity. The data were normalized by registering medians of each compound to equal one and normalizing each data point proportionately.

### Metabolomic data analysis

MetaboAnalyst 5.0 was used to analyze and visualize metabolomic data. All data that were input into MetaboAnalyst were $\log_{10}$-transformed, and autoscaled (mean-centered and divided by the standard deviation of each variable).

Unsupervised hierarchical clustering analysis (HCA) was performed on metabolomic data to visualize metabolic profiles as heatmaps and show relationships between global metabolic profiles and disease groups: endometrial cancer-All (grade 1/2 EEC and other endometrial cancer subtypes), endometrial hyperplasia, and benign conditions. For sample clustering, the Pearson distance measure and Ward linkage method was applied. HCA utilized the top 100 significant metabolites ($q < 0.05$) based on an analysis of variance (ANOVA) with false discovery rate (FDR) correction.

A two-sample $t$ test with FDR correction ($q < 0.05$) was performed to determine significant differences in metabolite levels between the disease groups. Fold change (FC) analysis was used to compare absolute value of change of the means of each metabolite between the two groups being investigated. The FC analysis utilizes data prior to data transformation and scaling. Data from FC analysis and $t$ test were combined to produce volcano plots depicting significantly up/downregulated metabolites ($q < 0.05$ and FC > 2) for comparison of two selected disease groups.

Enrichment analysis was performed to identify significantly altered metabolic pathways. The analysis was completed by comparing metabolite data to the Small Molecule Pathway Database metabolite set based on normal human metabolic pathways. Enrichment ratio and significance of enrichment of pathways were calculated based on number of metabolites detected within a specific pathway relative to number of known metabolites in that pathway. The algorithm also considered relative intensity of metabolites in each group. Despite being named pathway "enrichment" analysis, this method does not indicate upregulation or enrichment of pathways but determines pathways that are highly altered in the data. We may be able to draw conclusions about the direction of this alteration based upon individual metabolite levels.

Univariate receiver operating characteristic (ROC) analysis was performed to identify metabolic biomarkers that discriminate specific disease groups with high sensitivity and specificity. Mean levels of metabolites for each participant were used in the analyses. Strength of the discriminators was measured with AUC values. Metabolites with AUC greater than 0.8 or 0.9 were considered as good or excellent discriminators, respectively. Uncharacterized metabolites were excluded from ROC analysis.

Multivariate ROC curve analysis was performed using random forest algorithm and automated feature selection for sample classification. This analysis identifies the most important features, which are used to build predictive models distinguishing disease groups. Performance of predictive models was evaluated using Monte Carlo cross-validation and measured by AUC of multivariate ROC and the confusion matrix calculated at a probability threshold of 0.5.

To determine associations between metabolite levels and tumor characteristics, participants with endometrial cancer were stratified based on age (≥65 vs. <65), histological grade (grade 1/2 EEC vs. other endometrial cancer), MMR protein status (MMR deficient vs. MMR proficient), tumor size (>2 cm vs. ≤2 cm), and myometrial invasion (present vs. not present). Metabolite levels between those groups were compared using volcano plot analyses as described earlier. Spearman rank correlation analysis was performed to correlate metabolite levels to tumor size (measured in cm) and depth of myometrial invasion (measured in mm).

MetOrigin (freely available at: https://metorigin.met-bioinformatics.cn/home/) was used to identify putative metabolic origins of identified metabolites. MetOrigin determines whether the metabolite is from host, microbiome, or potential co-metabolism.

Differences in demographic, socioeconomic, and other participant-related variables between disease groups were tested using the Kruskal–Wallis test for continuous variables and Fisher's exact test for categorical variables.

### Data availability

The de-identified data generated in this study are available within the article and its supplementary data files. Raw data can be obtained upon reasonable request from the corresponding author.

## Results

### Study population

A total of 192 women undergoing hysterectomy were recruited and enrolled in this study. Women were classified into four groups: benign conditions ($n = 108$), endometrial hyperplasia ($n = 18$), grade 1/2 EEC ($n = 53$), and other endometrial cancer subtypes ($n = 13$) based on histopathological confirmation of biopsy samples. In some analyses, endometrial cancer is grouped as "endometrial cancer-All" ($n = 63$) and includes both grade 1/2 EEC and other endometrial cancer. Clinical and demographic information for this cohort was previously described (22). Key demographic information is displayed in **Table 1** and additional information relating to participant demographics and characteristics are included in Supplementary Table S3. Overall, participants had a mean age of 51 years, had a mean body mass index (BMI) of 34.8, and were mostly Caucasian (75%). Participants diagnosed with endometrial cancer were more often post-menopausal and on average were older with higher BMI (grade 1/2 EEC only) compared to participants with benign conditions. Further, information relating to abnormal uterine bleeding and post-menopausal bleeding presence in our participants can be found in Supplementary Table S4.

### Global metabolomics reveals distinct cervicovaginal metabolic profiles for endometrial cancer and benign participants

We used an untargeted global metabolomic approach and cervicovaginal sampling to assess the cervicovaginal metabolic profiles of women with endometrial cancer, hyperplasia, and benign conditions. We identified 920 metabolites across the CVL samples, belonging to lipid (25%), amino acid (22%), xenobiotic (19%), nucleotide (7%), peptide (4%), carbohydrate (4%), cofactors and vitamins (4%), energy (1%), and partially characterized/uncharacterized (13% and 1%, respectively) superpathways (**Fig. 1A**). Metabolite origin investigation revealed that metabolites detected in CVL samples likely originated from a mix of host (2.6%), microbiota (9%), co-metabolism (23%), and other (including drug, diet, environment, and unknown sources; 64%; Supplementary Fig. S1).

**Table 1.** Associations of key demographics with disease.

| | All ($n$ = 192) | Benign ($n$ = 108) | Hyperplasia ($n$ = 18) | Grade 1/2 EEC ($n$ = 53) | Other endometrial cancer ($n$ = 13) | $P$ value Overall | Paired comparison | |
|---|---|---|---|---|---|---|---|---|
| Age [mean (SD); $n$ = 192] | 51.02 (12.45) | 45.55 (10.01) | 54.11 (13.35) | 58.73 (11.82) | 60.77 (8.06) | <**0.0001** | Be vs. Hy | **0.007** |
| | | | | | | | Be vs. Grade 1/2 | <**0.0001** |
| | | | | | | | Be vs. Other | <**0.0001** |
| Race ($n$ = 190) | | | | | | 0.16 | | |
| American Indian/Alaskan | 15 (7.89) | 5 (4.67) | 1 (5.56) | 8 (15.38) | 1 (7.69) | | | |
| White/Caucasian | 142 (74.74) | 78 (72.90) | 16 (88.89) | 37 (71.15) | 11 (84.62) | | | |
| Black | 12 (6.32) | 11 (10.28) | 0 (0.00) | 1 (1.92) | 0 (0.00) | | | |
| All other | 21 (11.05) | 13 (12.15) | 1 (5.56) | 6 (11.54) | 1 (7.69) | | | |
| Ethnicity ($n$ = 191) | | | | | | 0.67 | | |
| Non-Hispanic | 141 (73.82) | 76 (70.37) | 14 (77.78) | 41 (78.85) | 10 (76.92) | | | |
| Hispanic | 50 (26.138) | 32 (29.63) | 4 (22.22) | 11 (21.15) | 3 (23.08) | | | |
| Alcohol use (current; $n$ = 175) | | | | | | 0.11 | | |
| Yes | 72 (41.41) | 50 (49.50) | 7 (43.75) | 11 (24.44) | 4 (30.77) | | | |
| No | 94 (69.23) | 47 (46.53) | 8 (50.00) | 30 (66.67) | 9 (69.23) | | | |
| Quit | 9 (5.14) | 4 (3.96) | 1 (6.25) | 4 (8.89) | 0 (0.00) | | | |
| Tobacco use (within last 6 months; $n$ = 184) | | | | | | 0.15 | | |
| Yes | 19 (10.33) | 14 (13.46) | 1 (5.56) | 3 (6.12) | 1 (7.69) | | | |
| No | 55 (29.89) | 34 (32.69) | 4 (22.22) | 13 (26.53) | 4 (30.77) | | | |
| Never | 89 (48.37) | 47 (45.19) | 12 (66.67) | 22 (44.90) | 8 (61.54) | | | |
| Quit | 21 (11.41) | 9 (8.65) | 1 (5.56) | 11 (22.45) | 0 (0.00) | | | |
| BMI [mean (SD); $n$ = 192] | 34.76 (10.16) | 30.63 (7.54) | 41.49 (7.45) | 40.29 (11.07) | 37.22 (12.76) | <**0.0001** | Be vs. Hy | <**0.0001** |
| | | | | | | | Be vs. Grade 1/2 | <**0.0001** |
| | | | | | | | Hy vs. Other | **0.005** |
| BMI ($n$ = 192) | | | | | | <**0.0001** | Be vs. Hy | <**0.0001** |
| <25 | 29 (15.38) | 23 (21.30) | 0 (0.00) | 4 (7.55) | 2 (15.38) | | Be vs. Grade 1/2 | <**0.0001** |
| 25–29 | 47 (24.38) | 38 (35.19) | 1 (5.56) | 6 (11.32) | 2 (15.38) | | | |
| 30–34 | 30 (15.63) | 19 (17.59) | 2 (11.11) | 6 (11.32) | 3 (23.08) | | | |
| ≥35 | 86 (44.79) | 28 (25.93) | 15 (83.33) | 37 (69.81) | 6 (46.15) | | | |
| Menopausal status ($n$ = 190) | | | | | | <**0.0001** | Be vs. Hy | <**0.0001** |
| Pre | 108 (56.84) | 89 (82.41) | 6 (33.33) | 12 (23.53) | 1 (7.69) | | Be vs. Grade 1/2 | <**0.0001** |
| Post | 82 (43.16) | 19 (17.59) | 12 (66.67) | 39 (76.47) | 12 (92.31) | | Be vs. Other | <**0.0001** |
| Combined contraceptives (use in past 6 months) | | | | | | | | |
| Hormonal ($n$ = 138) | | | | | | **0.01** | Be vs. Hy | **0.02** |
| Yes | 36 (26.09) | 26 (32.10) | 0 (0.00) | 5 (14.71) | 5 (45.45) | | Be vs. Grade 1/2 | **0.06** |
| No | 102 (73.91) | 55 (67.90) | 12 (100.00) | 29 (85.29) | 6 (54.55) | | Hy vs. Other | **0.01** |
| | | | | | | | Grade 1/2 vs. Other | **0.048** |
| Non-hormonal ($n$ = 64) | | | | | | 0.62 | | |
| Yes | 2 (3.13) | 1 (2.50) | 0 (0.00) | 1 (7.14) | 0 (0.00) | | | |
| No | 62 (96.88) | 39 (97.50 | 6 (100.00) | 13 (92.86) | 4 (100.00) | | | |
| Diabetes ($n$ = 192) | | | | | | **0.006** | Be vs. Grade 1/2 | **0.01** |
| Yes | 48 (25.00) | 22 (20.37) | 5 (27.78) | 21 (39.62) | 0 (0.00) | | Hy vs. Other | **0.05** |
| No | 144 (75.00) | 86 (79.63) | 13 (72.22) | 32 (60.38) | 13 (100.00) | | Grade 1/2 vs. Other | **0.01** |
| Hypertension ($n$ = 192) | | | | | | **0.002** | Be vs. Grade 1/2 | **0.001** |
| Yes | 65 (33.85) | 25 (23.15) | 6 (33.33) | 27 (50.94) | 7 (53.85 | | Be vs. Other | **0.04** |
| No | 127 (66.15) | 83 (76.85) | 12 (66.67) | 26 (49.06) | 6 (46.15) | | | |

NOTE: Values are $n$ (%) unless otherwise stated as mean (SD). $P$ values were calculated using the Kruskal–Wallis test for continuous variables and Fisher's exact test for categorical variables. Significant values ($p < 0.05$) are highlighted in bold.
Abbreviations: Be, benign; Hy, hyperplasia.

Using fold change analysis and $t$ tests with FDR correction, we identified significantly altered ($q < 0.05$ and FC > 2) metabolites in endometrial cancer groups compared to benign. Uncharacterized metabolites were not included in this analysis, as they have limited utilization. The endometrial cancer-All group had a total of 230 altered metabolites (167 downregulated and 63 upregulated) when compared to the benign group. The grade 1/2 EEC group had 204 altered metabolites (159 downregulated and 45 upregulated). The

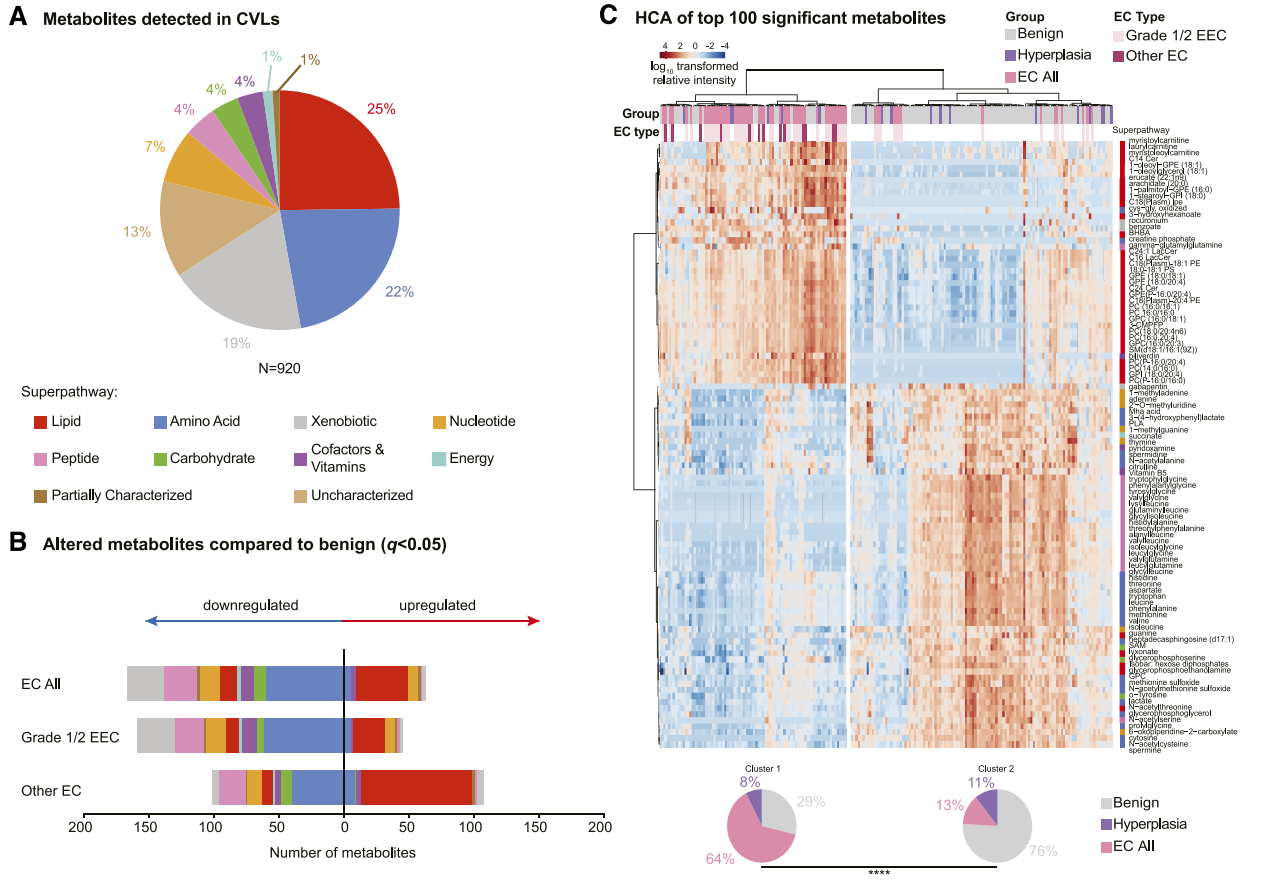


**Figure 1.**

Global metabolic analysis reveals differing profiles between patients with endometrial cancer (EC) and benign controls. **A,** Pie chart representing the proportions of different superpathways according to all detected metabolites ($n = 920$) in all samples ($n = 192$). **B,** Bar charts showing significantly altered ($q < 0.05$, FC > 2), FDR-corrected metabolites in endometrial cancer-All (EC All), grade 1/2 EEC, and other endometrial cancer (other EC) compared to benign controls. Color-coded by superpathway; not including uncharacterized metabolites. **C,** Hierarchical clustering analysis (HCA) heatmap representing the top 100 significant ($q < 0.05$) metabolites (filtered by ANOVA, with comparisons of benign, hyperplasia, and endometrial cancer-All). Pearson clustering and Ward linkage methods were used. Pie charts show proportion of disease groups in each cluster of samples. Metabolites are color-coded by superpathway, and samples are color-coded by disease group and endometrial cancer type. ****, $P < 0.001$. Uncharacterized metabolites were not included in this analysis.

other endometrial cancer group had 209 altered metabolites (101 downregulated and 108 upregulated). A large proportion of significantly upregulated metabolites in each endometrial cancer group were lipids: endometrial cancer-All had 41 (65.1% of all upregulated metabolites), grade 1/2 EEC had 25 (55.6%), and other endometrial cancer group had 86 (79.6%). Amino acids, peptides, and xenobiotics were significantly downregulated across endometrial cancer. In endometrial cancer-All, we identified 60 downregulated amino acids (35.9% all downregulated metabolites), 25 downregulated peptides (15%), and 29 downregulated xenobiotics (17.4%). In grade 1/2 EEC, there were 61 amino acids (38.4%), 23 peptides (14.5%), and 29 xenobiotics (18.2%). Finally, in other endometrial cancer, there were 40 amino acids (39.6%), 21 peptides (20.8%), and 5 xenobiotics (5%; **Fig. 1B**).

HCA stratified participants based on their metabolic profiles, producing a heatmap from the top 100 significant metabolites (ANOVA, $q < 0.05$; **Fig. 1C**). The analysis revealed two clusters were significantly ($P < 0.0001$) different in disease distribution (diseases grouped as benign, hyperplasia, and endometrial cancer-All and clusters based on dendogram). Hyperplasia was distributed between both clusters; due to this, hyperplasia did not take focus as a group in further analyses—we did not include participants with hyperplasia and analysis continued for benign ($n = 108$) versus grade 1/2 EEC ($n = 53$) and other endometrial cancer ($n = 13$). Cluster 1 consisted of 64% endometrial cancer, 29% benign, and 8% hyperplasia, and included all the other participants with endometrial cancer. Cluster 2 consisted of 76% benign, 13% endometrial cancer, and 11% hyperplasia. All the participants with endometrial cancer within cluster 2 were grade 1/2 EEC. Cluster 1 showed upregulated lipids and cluster 2 showed upregulated amino acids, peptides, and xenobiotics. This analysis revealed global metabolic profiles can successfully distinguish participants with endometrial cancer and benign conditions (**Fig. 1C**).

## Metabolic profiling reveals that endometrial cancer is associated with upregulation of lipids and downregulation of amino acids

Metabolomic investigation of CVL samples detected overall 228 lipids belonging to different classes: sterol lipids (14%), ketone

bodies (1%), glycerolipids (4%), glycerophospholipids (22%), ceramides (4%), other sphingolipids (21%), long-chain fatty acids (4%), and other fatty acids (30%; **Fig. 2A**). Lipids were significantly upregulated ($q < 0.05$ and FC > 2) in endometrial cancer participants compared to benign participants, particularly glycerophospholipids ($n = 10$ for endometrial cancer-All), other sphingolipids ($n = 5$ for endometrial cancer-All), and other fatty acids ($n = 11$ for endometrial cancer-All; **Fig. 2B**).

Also, overall 206 amino acids were detected in CVL samples, belonging to a number of different pathways: leucine, isoleucine, and valine metabolism (13%), histidine metabolism (11%), urea cycle (11%), methionine, cysteine, SAM, and laurine metabolism (10%), lysine metabolism (10%), tyrosine metabolism (8%), tryptophan metabolism (7%), polyamine metabolism (6%), glutamate metabolism (5%), glycine, serine, and threonine metabolism (4%), glutathione metabolism (4%), alanine and aspartate metabolism (4%), phenylalanine metabolism (3%), creatine metabolism (3%), and guanidino and acetamido metabolism (1%; **Fig. 2C**). Amino acids were significantly downregulated ($q < 0.05$ and FC > 2) in endometrial cancer compared to benign conditions, particularly methionine, cysteine, SAM, and laurine metabolism ($n = 11$ for endometrial cancer-All), tyrosine metabolism ($n = 7$ for endometrial cancer-All), and leucine, isoleucine, and valine metabolism ($n = 6$ for endometrial cancer-All; **Fig. 2D**).

### Endometrial cancer subtypes share many altered metabolites but have a number of uniquely altered metabolites

Significantly altered ($q < 0.05$ and FC > 2) metabolites were visualized on volcano plots to show up/downregulation. Comparison of grade 1/2 EEC to benign revealed 204 altered metabolites (45 upregulated and 159 downregulated). Comparison of other endometrial cancer subtypes to benign revealed 209 altered metabolites (108 upregulated and 101 downregulated; **Fig. 3A** and **B**). While both grade 1/2 EEC and other endometrial cancer have a similar number of altered metabolites when compared to benign, other endometrial cancer had more upregulated metabolites than grade 1/2 EEC, whereas grade 1/2 EEC had more downregulated metabolites than other endometrial cancer. The Venn diagram shows overlap and distinct populations of significantly altered metabolites between grade 1/2 EEC and other endometrial cancer. Grade 1/2 EEC has 70 uniquely altered metabolites (predominantly downregulated amino acids and xenobiotics) whereas other endometrial cancer has 84 uniquely altered metabolites (predominantly upregulated lipids). Both subgroups of endometrial cancer share 134 altered metabolites, mostly upregulated lipids ($n = 24$) and nucleotides ($n = 7$) and downregulated amino acids ($n = 40$), peptides ($n = 18$), and nucleotides ($n = 11$; **Fig. 3C**). Other endometrial cancer has a greater number of upregulated lipids than grade 1/2 compared to benign, particularly glycerophospholipids, other sphingolipids, and other fatty acids (**Fig. 3D**). Meanwhile, most downregulated amino acids are common among all subtypes of endometrial cancer, with grade 1/2 EEC having a small number of uniquely altered amino acids when compared to benign (**Fig. 3D**).

### Altered metabolic pathways in endometrial cancer

We performed pathway enrichment analysis to identify which metabolic pathways are significantly altered in the endometrial cancer-All group compared to the benign group. We identified 86 significantly ($P < 0.05$) altered pathways in endometrial cancer-All versus benign; the top 25 pathways are shown in Supplementary Fig. S2. Top pathways were associated with energy ($n = 5$), lipid ($n = 5$), amino acids ($n = 10$), cofactors and vitamins ($n = 3$), and nucleotide ($n = 2$). Among the most significantly altered pathways were energy pathways mitochondrial electron transport chain (METC; $P < 0.0001$) and glycerol phosphate shuttle ($P < 0.0001$); lipid pathways cardiolipin biosynthesis ($P < 0.0001$), *de novo* triacylglycerol biosynthesis ($P < 0.0001$) and glycerolipid metabolism ($P < 0.0001$); amino acid pathways arginine and proline metabolism ($P < 0.0001$), glutamate metabolism ($P < 0.0001$), histidine metabolism ($P < 0.0001$), and tryptophan metabolism ($P < 0.0001$); and nucleotide pathways pyrimidine metabolism ($P < 0.0001$) and purine metabolism ($P < 0.0001$).

### Metabolites in cervicovaginal lavages can discriminate endometrial cancer participants from benign participants

We performed ROC analysis to identify potential metabolic biomarkers that can distinguish participants with endometrial cancer from participants with benign conditions with high specificity and sensitivity. When comparing the endometrial cancer-All group to the benign group, there were 10 metabolites that reached a good biomarker threshold (AUC > 0.8): 6-oxopiperidine-2-carboxylate (AUC = 0.838), glycerophosphoethanolamine (GPEA; AUC = 0.37), glycerophosphocholine (GPC; AUC = 0.831), guanine (AUC = 0.826), cytosine (AUC = 0.819), glycerophosphoserine (AUC = 0.814), lyxonate (AUC = 0.810), prolylglycine (0.810), glycerophosphoglycerol (AUC = 0.807), and N-acetylserine (AUC = 0.807; **Fig. 4A**). When comparing only grade 1/2 EEC to benign, there were only six metabolites that reached the AUC > 0.8 threshold (all of which were among the 10 metabolites identified for endometrial cancer-All; **Fig. 4B**). ROC analysis comparing other endometrial cancer to benign conditions revealed many potential biomarkers [147 metabolites for other endometrial cancer subtypes, including biliverdin with an AUC > 0.9, considered an excellent discriminator (Supplementary Fig. S3)]. Overall, metabolites exhibited higher sensitivity and specificity for other endometrial cancer compared to grade 1/2 EEC (**Fig. 4B**). The top four biomarkers for endometrial cancer-All versus benign are shown as AUC plots, with individual AUC values for grade 1/2 EEC and other endometrial cancer depicted, which highlights these metabolites were more sensitive and specific for other endometrial cancer compared to grade 1/2 EEC (**Fig. 4C**). We performed statistical analysis with correction for BMI and age to ensure diagnostic markers are not signatures of age or obesity (Supplementary Table S5). We observed no loss in significance in any of the above diagnostic markers after correction.

### Machine learning–based multivariate models accurately predict endometrial cancer from benign conditions

We used a multivariate ROC approach based on a random forest algorithm to predict disease groups. This may be useful, as an individual biomarker may be elevated in other conditions; therefore, combination of multiple metabolites may exhibit higher sensitivity and specificity for endometrial cancer detection. A multivariate ROC analysis was conducted for endometrial cancer-All versus benign using a range of features from 5 to 100 metabolites, chosen by the machine learning algorithm to build multivariate models (**Fig. 5A**). The average AUC for all the number of features was >0.8 and therefore these models were considered good discriminators, with five-features and 100-feature models giving average AUCs of 0.826 and 0.884, respectively. A multivariate model with 25 metabolic features presents potential for a good diagnostic tool, with an AUC range of 0.800 to 0.951; predictive accuracy of each method

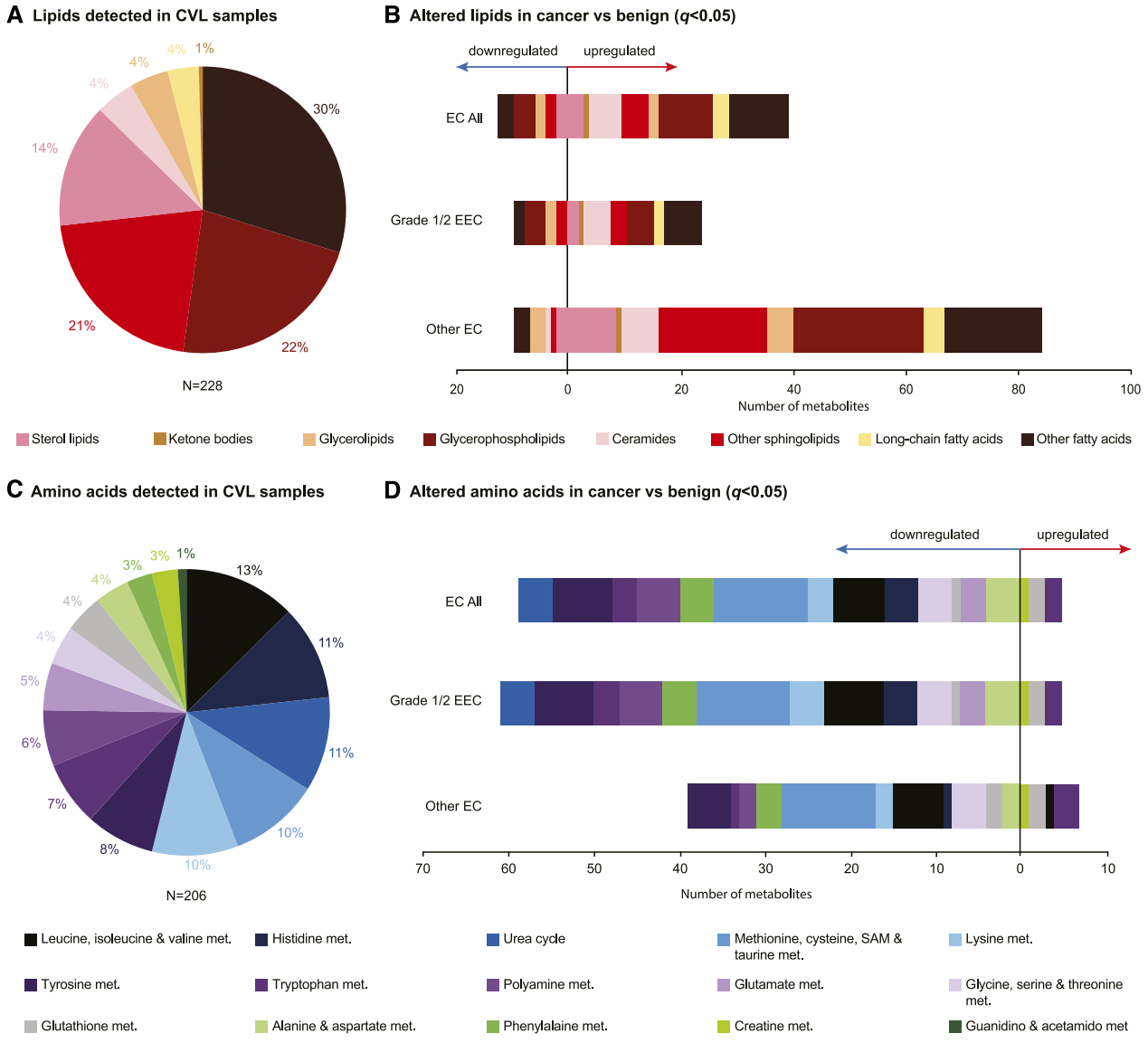


**Figure 2.**

Metabolic analysis reveals upregulation of lipids and downregulation of amino acids in endometrial cancer compared to benign conditions. **A,** Pie chart representing the proportions of different classes of lipids according to all detected lipids ($n = 228$) in all samples ($n = 192$). **B,** Bar charts showing significantly altered ($q < 0.05$, FC > 2), FDR-corrected lipids in endometrial cancer-All (EC All), grade 1/2 EEC, and other endometrial cancer (other EC) compared to benign controls. Color-coded by lipid class. **C,** Pie chart representing the proportions of different classes of amino acids according to all detected amino acids ($n = 206$) in all samples ($n = 192$). **D,** Bar charts showing significantly altered ($q < 0.05$, FC > 2), FDR-corrected amino acids in endometrial cancer-All, grade 1/2 EEC, and other endometrial cancer compared to benign controls. Color-coded by amino acid class.

remained consistent with 5 features having a predictive accuracy of 74.7% and 100 features having 78.9% (**Fig. 5A**). We focused on a 25-feature model based on the predictive accuracies and 25 being a manageable number of metabolites to be measured for a diagnostic test. The top 15 predictive features that were most frequently used to create the model by random forest included mostly lipids (GPEA, GPC, glycerophosphoserine, myristoleoylcarnitine, heptadecasphingosine, myristoylcarntine, 3-hydroxyhexonate, 1-palmitoyl-2-dihomo-linolenoyl-GPC (16:0/20:3n3 or 6), and palmitoleoylcarntine), and some nucleotides (guanine, AMP), amino acids (spermine and 6-oxopiperidine-2-carboxylate), peptides (prolylglycine), and cofactors and vitamins (pyridoxamine; **Fig. 5B**). Many metabolites used to build this multivariate model were also identified in the univariate ROC analyses, including guanine, GPEA, GPC, and prolylglycine. This model of 25 variables shows high predictive accuracy of 78.6% based on cross-validation, with many participants correctly classified into their disease group (**Fig. 5C**). A confusion matrix shows the times each sample obtained was classified correctly. For endometrial cancer-All, 83.3% ($n = 55$) of participants were correctly classified as endometrial cancer-All, and only 16.7% ($n = 11$) were incorrectly classified

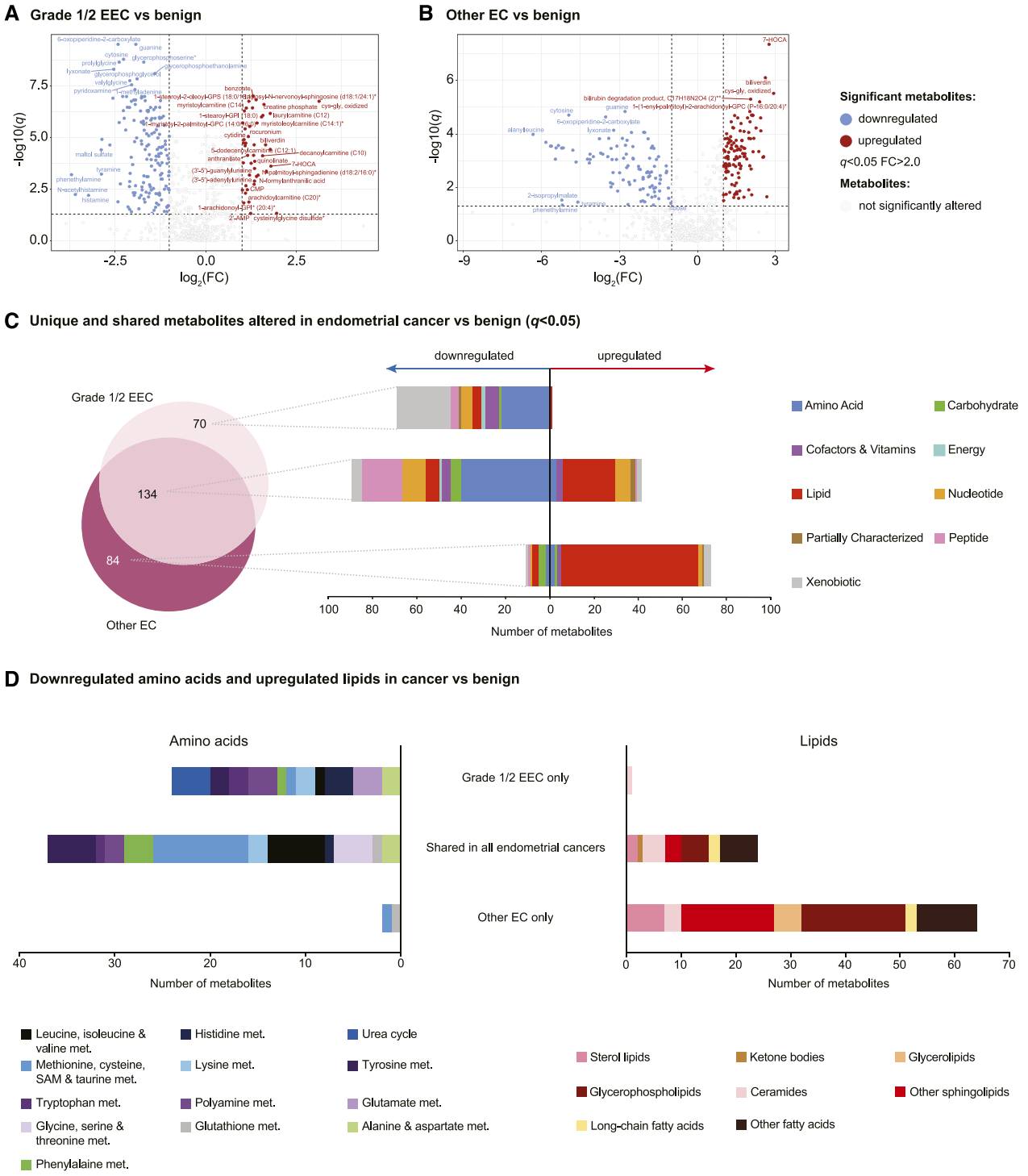


**Figure 3.**

Fold change and *t* test data reveal significant ($q < 0.05$, FC > 2) up/downregulation of metabolites in endometrial cancer compared to benign controls. **A** and **B,** Volcano plots showing FDR-corrected, significantly altered ($q < 0.05$, FC > 2) metabolites in grade 1/2 EEC (**A**), and other endometrial cancer (other EC) (**B**) compared to benign controls. Color-coded by significance and up/downregulation. Top altered metabolites are labeled. Uncharacterized metabolites not included in this analysis. **C,** Venn diagram compares all significantly altered ($q < 0.05$, FC > 2) metabolites in grade 1/2 EEC and other endometrial cancer (other EC) compared to benign controls. Bar plot demonstrates the superpathway profiles of each section from the Venn diagram. **D,** Bar charts representing downregulated amino acid and upregulated lipid classes unique to each cancer type and shared between all endometrial cancers.

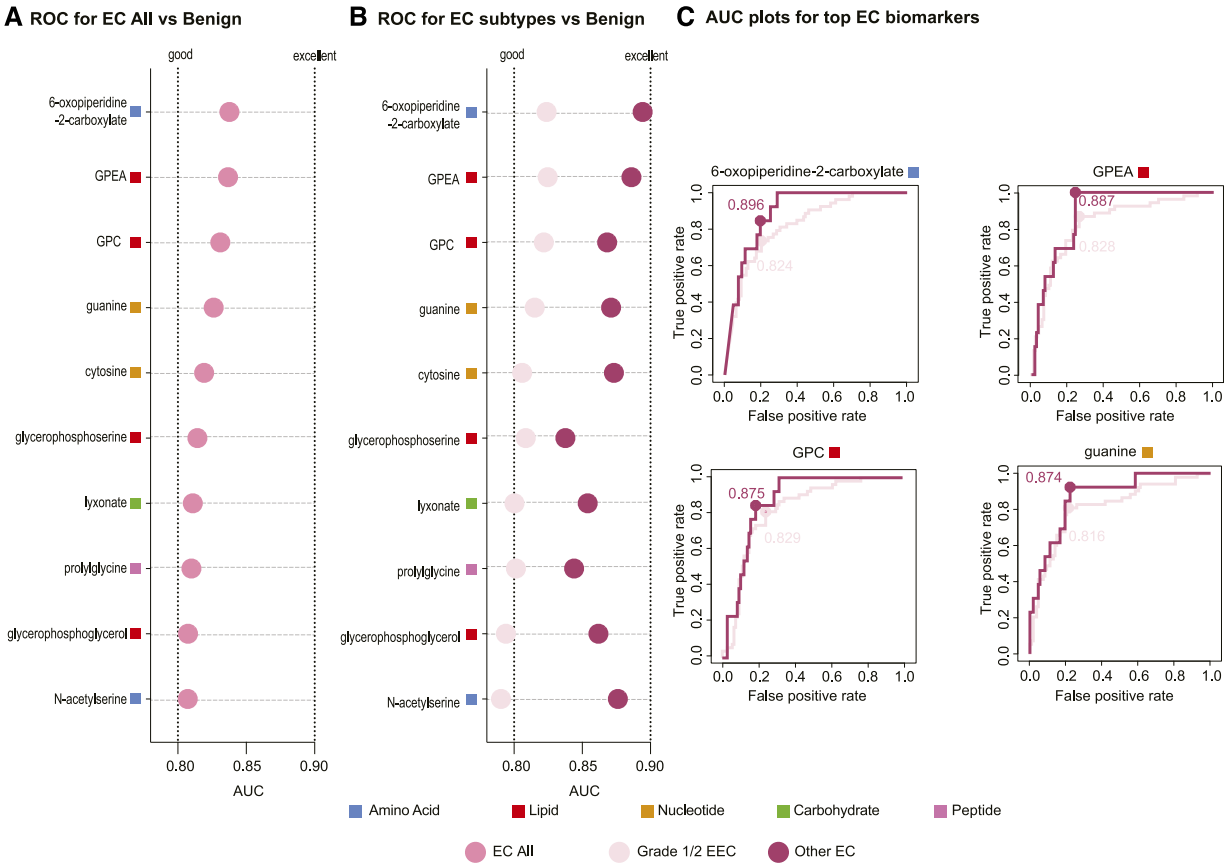


**Figure 4.**

Receiver operating characteristic (ROC) analysis reveals a multitude of potential biomarkers to detect patients with endometrial cancer from benign controls. **A,** Scatter plot showing 10 metabolites had an AUC > 0.8 (good biomarker threshold) for endometrial cancer-All (EC All) vs. benign controls. Metabolites color-coded by superpathway. Uncharacterized metabolites not included in analysis. **B,** Scatter plot showing the AUC values of the 10 metabolites from panel **A** for grade 1/2 EEC and other endometrial cancer (other EC) vs. benign controls. Metabolites color-coded by superpathway. **C,** AUC plots for the top four potential endometrial cancer-All biomarkers showing the AUC values for grade 1/2 EEC and other endometrial cancer vs. benign controls.

as benign. For benign participants, 79.6% ($n$ = 86) were correctly classified as benign, and 20.4% ($n$ = 22) were incorrectly classified as endometrial cancer-All (**Fig. 5D**).

## Cervicovaginal metabolite levels are reflective of tumor characteristics

We grouped endometrial cancer participants based on tumor characteristics: histological grade (other endometrial cancer vs. grade 1/2 EEC), MMR status (MMR deficient vs. MMR proficient), tumor size (>2 cm vs. ≤2 cm), and myometrial invasion (present vs. not present). In addition, we stratified participants with endometrial cancer based on age (≥65 years vs. <65 years), as increased age is a risk factor of endometrial cancer. Volcano plots show significantly up/downregulated ($P$ < 0.05 and FC > 2) metabolites associated with each tumor characteristic (**Fig. 6A**). For age, we identified one downregulated metabolite (nicotinate ribonucleoside) and one upregulated metabolite (N6-methyllysine). For histological grade, we identified 25 upregulated metabolites and two downregulated metabolites [2-methylbutyrylcarnitine (C5) and homocysteine]. For MMR status, we identified three upregulated [decanoylcarnitine (C10), laurylcarnitine (C12), and octonolycarnitine (C8)] and eight downregulated. For myometrial invasion, we identified nine downregulated and six upregulated, including many lipids. Finally, for tumor size, we identified 25 downregulated and 53 upregulated, 56% of which were lipids ($n$ = 44). In addition, Spearman rank correlation analysis was used to show the strength of relationship between individual metabolite levels and tumor size (measured in cm) and depth of myometrial invasion (measured in mm). The top 10 metabolites identified that were significantly correlated with tumor size and/or myometrial invasion (with a mix of some positively and some negatively correlating) included amino acids, carbohydrates, lipids, and nucleotides. 5-Methyluridine, AMP, C16 Ceramide (Cer), and uridine all correlated with both tumor size and myometrial invasion (mix of negative and positive correlation; **Fig. 6B**). All results from Spearman rank analysis are in Supplementary Table S6. The Venn diagram illustrates number of metabolites that were unique or shared between tumor characteristics (**Fig. 6C**). Age did not have any shared altered metabolites with any tumor characteristics investigated. Myometrial invasion and tumor size shared nine metabolites [AMP, 2,3-diphosphoglycerate, C16DH Cer, argininate, C16 Cer, (3′–5′)-cytidyluridine, C14 Cer, N-stearoyl-sphingosine (d18:1/18:0), 1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0), PC-(P-16:0/16:0)], MMR status and tumor size shared adenosine only, and histological grade and tumor size shared four metabolites

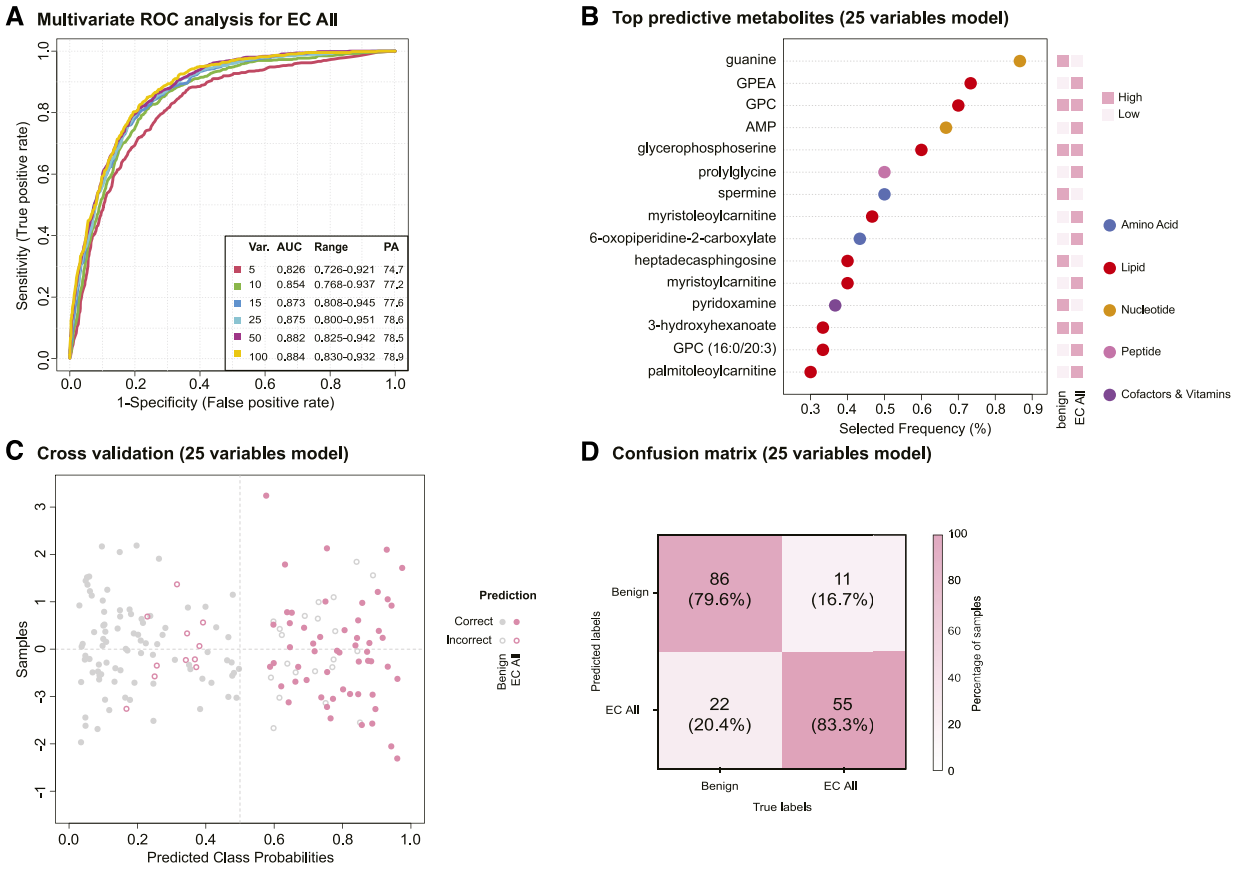


**Figure 5.**

Multivariate ROC analysis reveals potential for a multiple metabolites test to distinguish patients with endometrial cancer from benign controls. **A,** AUC plot from multivariate analysis, color-coded by number of metabolites. Metabolites were chosen for analysis by random forest machine learning. Uncharacterized metabolites were not included in analysis. **B,** The top 15 selected metabolites (based on random forest) and their selected frequency. Color-coded by superpathway. Squares represent the relative levels of each metabolite in endometrial cancer-All (EC All) and benign. **C,** Cross validation plot demonstrating the ability of multivariate ROC to predict disease group. **D,** Confusion matrix demonstrating the percentage of samples predicted correctly/incorrectly. Color-coded by percentage of total samples per disease group. AUC, area under the curve; Range, AUC range; PA, predictive accuracy; Var., number of variables (metabolites).

[homocysteine, 1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2), 3-hydroxybutyrate, and 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4), PC-(P-16:0/20:4)].

## Discussion

This cross-sectional study investigated global metabolic profiles of CVL samples collected from 192 women undergoing hysterectomy for benign or malignant indications. Participants were grouped based on histology, benign conditions ($n = 108$), endometrial hyperplasia ($n = 18$), grade 1/2 EEC ($n = 53$), and other endometrial cancer ($n = 13$). Utilizing this cohort and an untargeted metabolomics approach, we identified a unique cervicovaginal metabolic profile for endometrial cancer compared to benign conditions. CVL sampling has been shown by our and others' previous research to have utility for investigating pathophysiological mechanisms in a multitude of different gynecologic conditions (18–24). In addition, CVLs have demonstrated utility in biomarker discovery and predictive accuracy similar to more invasive sampling techniques such as biopsy and blood draws (18–23).

To our knowledge, this is the largest comprehensive metabolic analysis studying endometrial cancer in the context of CVL sampling and untargeted high-performance liquid chromatography–mass spectrometry, and largest metabolic profiling of endometrial cancer using noninvasive sampling. We detected levels of over 900 metabolites and showed a distinct alteration of metabolic profiles in participants with cancer versus benign controls, including significant ($q < 0.05$) upregulation of lipids and downregulation of amino acids, peptides, and nucleotides.

Our previous work utilizing CVL metabolomics within cervical cancer revealed depletion of amino acids and enrichment of lipids (particularly sphingolipids and glycerophospholipids) associated with cancer (18). Upregulation of glycerophospholipids has also been investigated in endometrial cancer by other groups, showing glycerophospholipid levels in endometrial tissue may be indicative of endometrial cancer (29).

Altered lipid metabolism has been previously linked to cancer, as cancer cells require lipids for growth, energy, and survival (30). Lipid upregulation has been associated with cancer progression, including metastasis (31). Previously, lipid levels have been shown

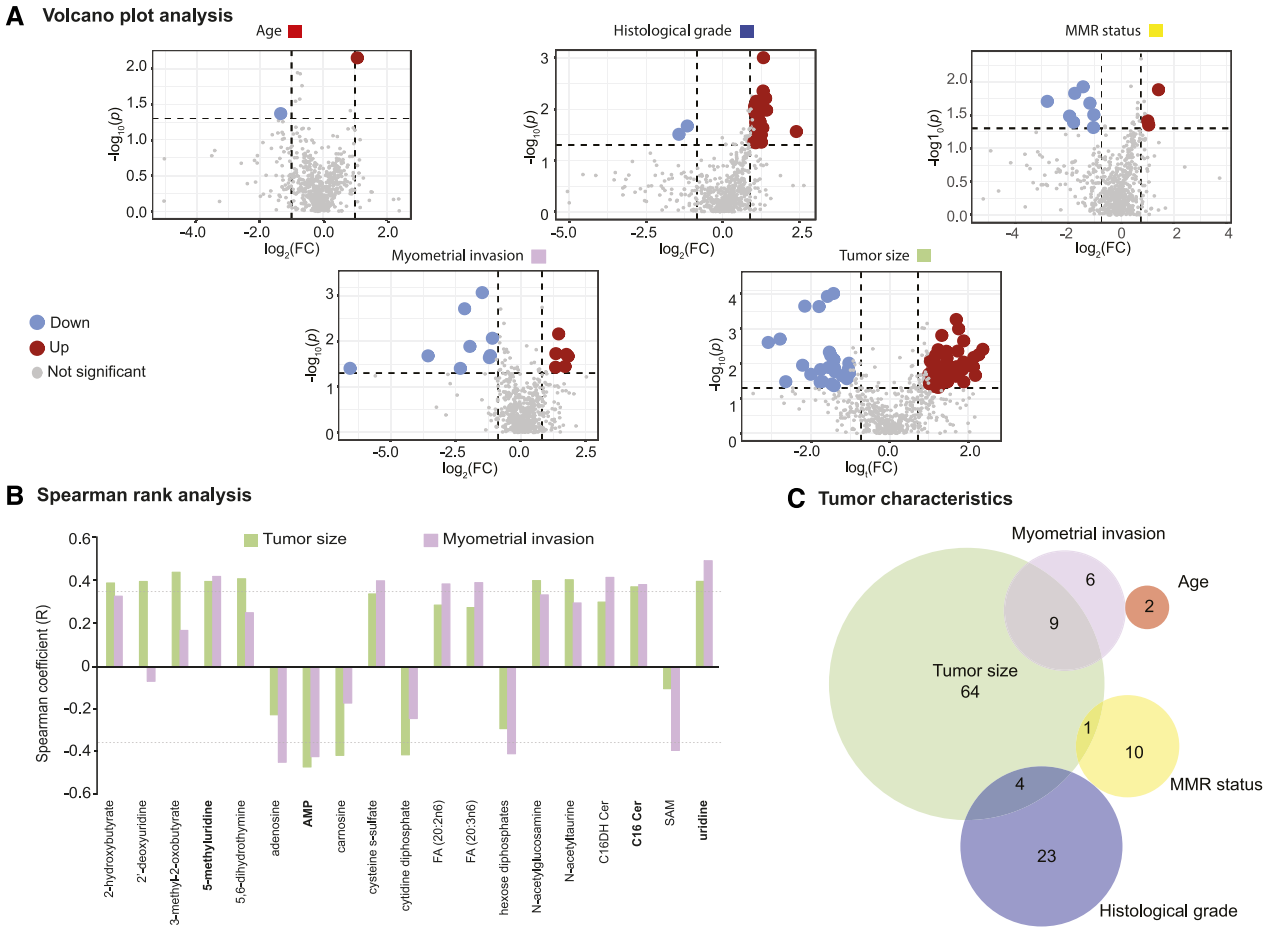


**Figure 6.**

Analysis of pathology data reveals relationship between metabolites and tumor characteristics. **A,** Fold change and *t* test data combined to produce volcano plots show significantly ($P < 0.05$) up/downregulated (FC > 2) metabolites associated with histological grade (other endometrial cancer vs. grade 1/2 EEC), MMR status (MMR deficient vs. MMR proficient), myometrial invasion (present vs. not present), and tumor size (>2 cm vs. ≤2 cm). **B,** Spearman correlation analysis shows top 20 significantly ($P < 0.05$) correlated metabolites with increased tumor size and increased depth of myometrial invasion. **C,** Venn diagram displaying number of significantly altered ($P < 0.05$, FC > 2) metabolites that are unique or shared among the different tumor characteristics.

to be upregulated in blood samples from patients with endometrial cancer (32), highlighting comparability of CVLs against other sampling techniques. Aberrant lipid metabolism is suggested as not only a factor involved in cancer development and progression but also the increase in lipid synthesis may be a cancer survival mechanism (33). Our metabolomics analysis of the CV microenvironment revealed enrichment of fatty acids, glycerophospholipids, and other sphingolipids in endometrial cancer. Fatty acids have been associated with cancer through their role in cellular proliferation (34), providing an energy source (35), and mediating metastasis development (35). Glycerophospholipids are promotors of cellular proliferation, tumorigenesis, and cancer survival (36). Finally, sphingolipids are implicated in cancer due to their ability to invade into neighboring tissue (37, 38), promote angiogenesis and metastasis (37, 38), and accelerate tumor growth (37, 38). Therefore, lipids may play a role in carcinogenesis, as well as in disease progression and severity.

Previous work has linked alterations in amino acid metabolism to cancer; it is theorized that cancer cells use amino acids as an alternate energy source (39), to provide proliferative ability (40), and to maintain redox homeostasis (40). Previous studies, utilizing human samples and animal models, have shown downregulation of amino acids in endometrial cancer (41); our results align with this finding. Pathway analysis identified significant alterations in a number of amino acid pathways. Altered amino acid pathways included metabolism of arginine and proline, glutamate, histidine, and tryptophan. Proline can protect cells from oxidative stress damage and therefore can be utilized by cancers to promote growth (42). Furthermore, previous work has shown the potential of proline as a plasma biomarker for endometrial cancer (43). The literature shows glutamate enhances cancer cell proliferation in endometrial and breast cancer *in vitro* (44, 45). Histidine metabolism was highly altered, similarly to our work using CVLs from participants with adenomyosis, relating to uterine contractility (20). Tryptophan metabolism has already been well established to be increased in cancer, due to its ability to aid immune suppression (46).

Our results show while all endometrial cancer types share most altered metabolites, there are also unique metabolic signatures

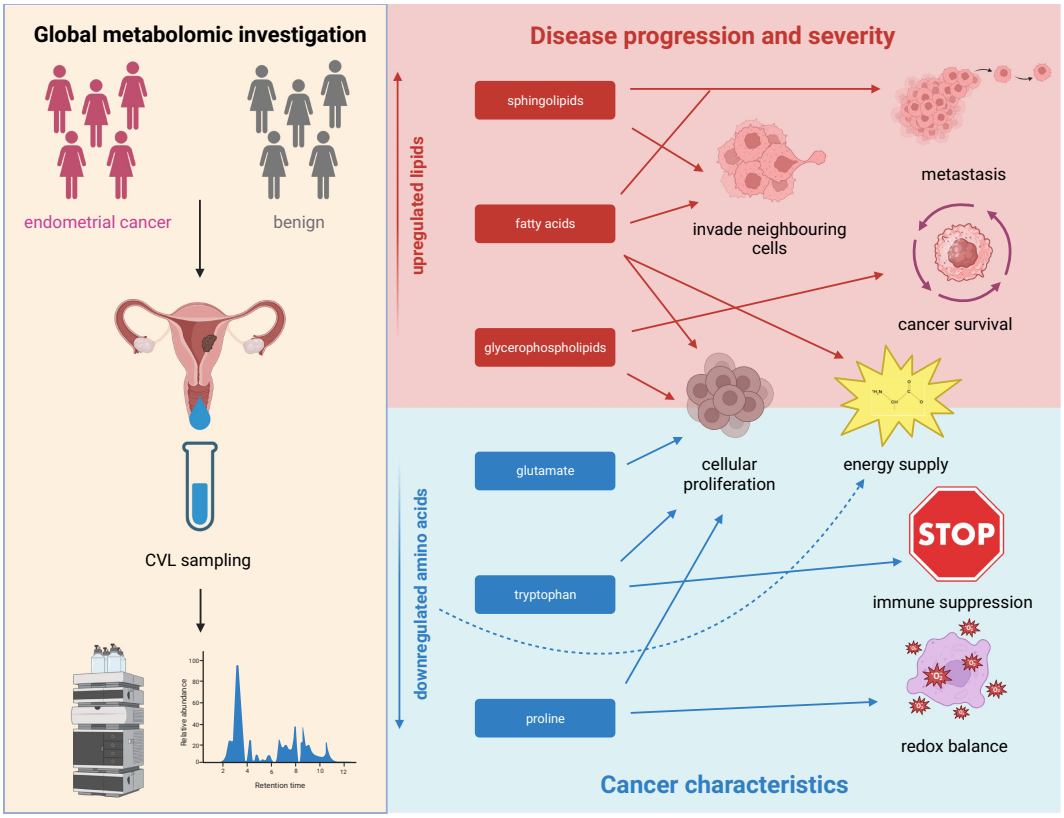


**Figure 7.**
Graphical summary of metabolomic investigation into endometrial cancer utilizing cervicovaginal lavage sampling. Overview of dysregulated metabolites and associations with pathophysiological mechanisms related to disease. Solid arrows represent associated links between metabolites and mechanisms, dashed arrows represent tentative links between metabolites and mechanisms. (Created with BioRender.com.)

associated with histological grade and type. Grade 1/2 EEC has more downregulated amino acids, whereas other endometrial cancer has unique upregulation, particularly of glycerophospholipids, other sphingolipids, and fatty acids. The other endometrial cancer group in this cohort consists of grade 3 EECs and other histological subtypes, such as serous carcinoma, which are known to be more aggressive forms of endometrial cancer (47). Yang and colleagues (48) have previously described aggressive forms of liver cancer that create a lipid-enriched microenvironment influencing immune reprogramming and allowing liver cancer to metastasize. Between the two groups of endometrial cancer within this study, grade 1/2 EEC and other endometrial cancer, we identified both an overlap and distinct profile of metabolic signatures, with other endometrial cancer displaying a distinct upregulation of lipids, which may correspond to its aggressiveness.

Pathway analysis may provide mechanistic insights into the pathophysiology of endometrial cancer. The most highly altered pathways for endometrial cancer-All in our study were energy-related pathways, METC, and the glycerol phosphate shuttle. Yao and colleagues (49) have previously revealed glycerol phosphate shuttles in kidney cancer play a role in redox balance as well as sustaining lipid synthesis, linking dysregulation of this pathway to the enriched lipid microenvironment in endometrial cancer. METC has been shown to be altered in lung cancer (50), causing reprogramming of cellular metabolism, which is a hallmark of cancer (51). Tumor environments require higher demands of energy to complete cellular processes for sustained upregulated cellular proliferation (52).

Cardiolipin biosynthesis has not previously been linked specifically to endometrial cancer; however, it was highly altered in our dataset. Guri and colleagues (53) linked elevated cardiolipin levels to liver tumorigenesis in mouse models, stating that cardiolipin may represent essential lipids required for cell growth. Triacylglycerol pathways were also altered in endometrial cancer, and previous work has shown the potential link with upregulated serum triacylglycerols and risk of colorectal cancer (54). Yang and colleagues (55) discussed metabolic syndrome and risk of endometrial cancer, stating increased levels of triglyceride (triacylglycerols) in serum is a factor of metabolic syndrome, highlighting that the altered triacylglycerol synthesis found within our endometrial cancer cohort compliments previous work describing risk of endometrial cancer development. In our study, endometrial cancer participants were more frequently obese than benign participants; therefore, this can be a confounder in our results. However, when adjusting for age and BMI, statistical significance was not altered for any metabolites identified in the biomarker discovery analysis. Furthermore, glycerolipid metabolism was highly altered in our participants with endometrial cancer. Past studies have shown how glycerolipid pathways are involved in triacylglycerol production, and this

metabolic network promotes cancer survival in a nutrient-deficient environment (56). Overall, there is strong dysregulation of lipid levels (particularly glycerophospholipids, other sphingolipids, and other fatty acids) and pathways within the endometrial cancer population, giving rise to reprogramming of cellular metabolism and aiding cancer survival.

Pyrimidine metabolism is altered within endometrial cancer, consistent with our previous investigation of adenomyosis, and may reflect the hyperproliferative nature of the endometrium in both disease states. Primarily, this pathway is involved in nucleic acid synthesis (57), which is required for excessive cellular proliferation. Overall, changes in metabolic pathways identified in endometrial cancer contribute to cancer growth and survival.

By implementing biomarker discovery tools, our work has identified a number of key metabolites to serve as potential biomarkers detectable in CVL samples. These included a multitude of metabolites from different superfamilies and pathways, including lipids (GPEA, GPC, glycerophosphoserine, and glycerophosphoglycerol), amino acids (6-oxopiperidine-2-carboxylate and N-acetylserine), and nucleotides (guanine and cytosine). Of these, one metabolite with a high specificity and sensitivity for both grade 1/2 EEC and other endometrial cancer types was GPEA. This metabolite directly relates to a state of altered lipid metabolism due to involvement in cell membrane structure, cellular interactions, and cellular signaling (58). Therefore, alterations in GPEA levels, and thus altered lipid metabolism, link our data to previous research associating aberrant lipid levels to endometrial cancer (29, 32). For grade 3 EEC and other endometrial cancer subtypes, biliverdin was considered an excellent biomarker candidate. Biliverdin is a metabolite produced from breakdown of heme; previous research has shown heme to be a potential biomarker for endometrial cancer in serum samples (59). In previous work, Genkinger and colleagues (60) associated heme dysregulation with endometrial cancer risk, stating this may be due to heme-induced oxidative stress causing DNA damage.

Results from our multibiomarker prediction models, which provided strong sensitivity and specificity for endometrial cancer, give rise to the potential for diagnosis of endometrial cancer via more comfortable and acceptable means for patients compared to current techniques (such as biopsy or D&C). A combination of 25 metabolites identified by a machine-learning algorithm provides a strong basis for the selection of key biomarker candidates to detect endometrial cancer. This model does not automatically consider demographic information such as age and BMI; therefore, we completed correction for age and BMI, and all of the biomarkers found significant in this model remained significant. Future studies are needed to validate our findings in larger and diverse cohorts. This multibiomarker panel approach also provides an advantage of not relying on levels of a singular biomarker for diagnosis; individual metabolites may be increased for a multitude of reasons, including non-malignant diseases. For example, our results show that metabolites relating to tryptophan and histidine metabolism are enriched in both endometrial cancer and adenomyosis (a non-malignant gynecologic condition affecting the endometrium; ref. 20).

Remarkably, we demonstrated metabolites detected in CVL samples are reflective of tumor characteristics, such as tumor size, myometrial invasion, and MMR status. To the best of our knowledge, this is the first metabolomics study in endometrial cancer analyzing relationships between metabolite levels in noninvasive samples and tumor characteristics. These results are crucial in highlighting that not only do cervicovaginal metabolites hold potential for diagnosis of endometrial cancer, but they also hold prognostic value, since these characteristics (tumor size, myometrial invasion, histological grade, MMR status, as well as age) are used frequently by clinicians to assess risk stratification for operation, as well as deciding whether a total hysterectomy is required to provide the best possible outcome for participants. Our results indicate that metabolite levels are indicative of tumor characteristics, particularly tumor size and myometrial invasion, particularly pyrimidine and purine metabolites (2′-deoxyuridine, 5-methyluridine, 5,6-dihydrothymine, adenosine, AMP, cytidine diphosphate, and uridine). Previous work has shown these metabolite groups were related to excess cellular proliferation in adenomyosis (20). Furthermore, a number of these metabolites [PC (P-16:0/20:4), PC (P-16:0/16:0), and uridine] were also potential biomarkers for cervical cancer in our previous study (18), highlighting potential links between cervical and endometrial cancer progression. The associations observed with tumor characteristics are an important finding in our research, warranting further investigation in future cohorts.

This study provides a foundation for metabolic investigation of endometrial cancer, being the largest global metabolomics investigation of the CV microenvironment. We had a large cohort size with 192 hysterectomy participants, including 53 participants with the most common grade 1/2 EEC. In addition, we were able to enroll a small cohort (13 participants) with other, more aggressive, but less common, endometrial cancer subtypes. We were able to identify biomarker candidates for endometrioid subtypes as well as more aggressive other subtypes.

There are a few limitations to our preliminary study, but all of which can hopefully be validated in future cohorts. We did not have participants with very advanced stage cancers, with many of our participants being in early stages of endometrial cancer, but this is a strength, as we were able to detect distinguishing metabolites in the lavages for mostly early stage I tumors. Our control group consisted of participants with benign gynecologic conditions, and women with benign conditions often have similar symptoms to those with endometrial cancer (i.e., abnormal uterine bleeding); therefore, it is important to be able to distinguish between benign and malignant groups. We excluded women based on sexually transmitted infections; however, this did not include HPV, as we did not have access to such data—therefore it will be crucial in the future to account for this and check the validity of results. Further, some identified metabolites were uncharacterized due to lack of appropriate library standards. This limits their current ability to give us insight into endometrial cancer pathology; however, these metabolites still can exhibit diagnostic value and may be characterized in the future with rapid advances in the metabolomics field. The CVL samples in this study were collected by a physician in the operating room prior to surgery; however, CVL samples do not need to be collected by a medical professional and can be collected by oneself, thereby opening the scope for development of easily accessible tests. This sampling method does not replace traditional biopsy techniques but begins the journey towards improved detection, diagnosis, and treatment of endometrial cancer through more accessible and less invasive means.

Initial investigation in this article highlighted that some metabolites detected were likely originated from microbiota and not from the host. While we did not have the capacity to investigate this interaction further within this article, it has highlighted a need for future studies investigating the impact of the human microbiome on metabolism in the context of endometrial cancer and other gynecologic malignancies, as well as multiomics studies to yield insights into host-microbiome interplay in health and disease.

### Conclusions

To summarize, by metabolomic investigation of CVL samples we were able to effectively distinguish participants based on disease status and endometrial cancer subtype. These metabolites informed us of metabolic pathways and pathophysiological processes altered within endometrial cancer (**Fig. 7**). Key to our findings is that downregulation of amino acids observed within endometrial cancer is likely a result of general cancer characteristics, while upregulation of lipids aligns with disease progression and severity. These metabolites, both singular and combined, were highly predictive of cancer and provide potential diagnostic capabilities via CVL sampling. Combination of metabolites, using multivariate biomarker discovery analysis, showed that a diagnostic model of 25 metabolites provided a high correct prediction rate of disease. Strikingly, these metabolites were predictive of tumor characteristics, disease severity, and histological grade, providing potential prognostic capability and risk stratification for treatment planning using cervicovaginal sampling.

## Authors' Disclosures

P. Łaniewski reports grants from National Institutes of Health during the conduct of the study; in addition, P. Łaniewski has a patent for Metabolic Screening Methods for Endometrial Cancer pending. J. Mourad reports personal fees from INTUITIVE SURGICAL and HOLOGIC outside the submitted work. L. Willmott reports other support from AstraZeneca, Eisai, Immunogen, Merck, and Seagen outside the submitted work. D.M. Chase reports personal fees from Merck, Eisai, GSK, AstraZeneca, and Immunogen outside the submitted work. D.J. Roe reports grants from National Cancer Institute during the conduct of the study. M.M. Herbst-Kralovetz reports grants from Mary Kay Foundation, Valley Research Partnership, Phoenix Friends Foundation, National Cancer Institute of the National Institutes of Health, and Arizona Biomedical Research Center during the conduct of the study; in addition, M.M. Herbst-Kralovetz has a patent for Metabolic Screening Methods for Endometrial Cancer pending. No disclosures were reported by the other authors.

## Authors' Contributions

**G.M. Lorentzen:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft. **P. Łaniewski:** Conceptualization, data curation, formal analysis, supervision, validation, investigation, visualization, methodology, writing–review and editing. **H. Cui:** Formal analysis, writing–review and editing. **N.D. Mahnert:** Writing–review and editing. **J. Mourad:** Writing–review and editing. **M.P. Borst:** Writing–review and editing. **L. Willmott:** Writing–review and editing. **D.M. Chase:** Validation, writing–review and editing. **D.J. Roe:** Formal analysis, writing–review and editing. **M.M. Herbst-Kralovetz:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, project administration, writing–review and editing.

## Acknowledgments

We would like to thank the participants enrolled in the study and acknowledge Regina Montero and Elisa Martinez for their kind assistance in participant recruitment, sample, and clinical data collection. We would also like to thank Dr. Nicole Jimenez for her kind assistance in exploring the metabolic origin of our metabolites. This study was funded by the Mary Kay Foundation (M.M. Herbst-Kralovetz), the Valley Research Partnership (D.M. Chase and M.M. Herbst-Kralovetz), the Phoenix Friends Foundation (M.M. Herbst-Kralovetz), the National Cancer Institute of the National Institutes of Health under award number P30 CA023074 (M.M. Herbst-Kralovetz), and the Arizona Biomedical Research Center (ABRC) Arizona Investigator Grant #39084114 (P. Łaniewski and M.M. Herbst-Kralovetz) and support provided to P.L. through the Guiding U54 Investigator Development to Sustainability (GUIDeS) program under the award for the Partnership of Native American Cancer Prevention funded by the National Cancer Institute of the National Institutes for Health award number U54CA143924 (M.M. Herbst-Kralovetz)

## Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received September 29, 2023; revised February 2, 2024; accepted April 26, 2024; published first April 30, 2024.

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