Immunometabolic profiling of cervicovaginal lavages identifies key signatures associated with adenomyosis

Highlights

- Pyrimidine, carnitine, and histidine/histamine pathways are enriched in adenomyosis
- Immunometabolic profiles associate with adenomyosis and immune dysregulation
- Immunometabolic alterations allow a state of excess proliferation and fibrosis
- Cervicovaginal lavages offer non-invasive sampling with potential diagnostic value
SUMMARY
Adenomyosis is a burdensome gynecologic condition that is associated with pelvic pain, dysmenorrhea, and abnormal uterine bleeding, leading to a negative impact on quality of life; and yet is often left undiagnosed. We recruited 108 women undergoing hysterectomy for benign gynecologic conditions and collected non-invasive cervicovaginal lavage samples for immunometabolic profiling. Patients were grouped according to adenomyosis status. We investigated the levels of 72 soluble immune proteins and >900 metabolites using multiplex immunoassays and an untargeted global metabolomics platform. There were statistically significant alterations in the levels of several immune proteins and a large quantity of metabolites, particularly cytokines related to type II immunity and amino acids, respectively. Enrichment analysis revealed that pyrimidine metabolism, carnitine synthesis, and histidine/histamine metabolism were significantly upregulated pathways in adenomyosis. This study demonstrates utility of non-invasive sampling combined with immunometabolic profiling for adenomyosis detection and a greater pathophysiological understanding of this enigmatic condition.

INTRODUCTION
Adenomyosis is a benign gynecologic condition characterized by the ectopic growth of endometrial tissue in the myometrium. It is a highly prevalent condition, with an estimated occurrence of 30–35% in symptomatic patients. Symptoms include dysmenorrhea, chronic pelvic pain, and abnormal uterine bleeding; however, it is estimated that a third of cases are asymptomatic. Adenomyosis is also associated with infertility, as well as a number of negative obstetric outcomes, including: pre-eclampsia, pre-term birth, fetal malpresentation, and post-partum hemorrhage. Current definitive diagnostic tools for adenomyosis rely on histopathology testing after hysterectomy. Imaging techniques are currently being explored for diagnosis; however, this approach requires further investigation and optimization for reliable results. Reliance on hysterectomy for diagnosis may introduce a bias, as women willing to undergo hysterectomy are more often older and have higher parity; therefore, adenomyosis prevalence is likely to be higher. Adenomyosis has a high co-occurrence rate with other gynecologic conditions, particularly endometriosis and fibroids. The combination of bothersome symptoms, comorbidity, and the lack of accurate diagnostic tools leads to a poor quality of life for these patients. Thus, there is an urgent need for improved understanding of the pathophysiology of the condition, and the development of robust and accessible diagnostic tools to detect adenomyosis.

There is currently very limited research into potential biomarkers for adenomyosis, particularly those detectable through non-invasive methods. The majority of previous studies focused on the serum protein biomarkers, such as cancer antigen 125 (CA125) or human epididymis protein 4 (HE4), but these biomarkers have not been adapted for clinical use for adenomyosis. Serum levels of CA125 have been shown to be increased in adenomyosis patients; however, this biomarker is typically elevated in women with other conditions, such as endometriosis, or endometrial and ovarian cancers, which limits its diagnostic value.
In this study, we utilize multiplex immunoassay and global metabolomics platforms coupled with non-invasive CVL sampling, to identify novel protein and metabolic biomarkers for adenomyosis detection. In addition, we aimed to better understand the pathophysiological processes behind adenomyosis. Through this acquired knowledge, there is potential for improved detection and treatment of adenomyosis.

RESULTS
Study sample

In this study, we investigated immunoproteomic and metabolic differences between patients with and without adenomyosis. We recruited women undergoing hysterectomy for benign conditions (n = 108) and collected non-invasive CVL samples for downstream analysis. The women were stratified according to whether they were diagnosed with adenomyosis (n = 46) or no adenomyosis (n = 62) based on histopathological confirmation post-hysterectomy.

Clinical and demographic information for this cohort is reported in Table 1. The mean age of patients enrolled was 45.6 years, with no significant difference between groups (p = 0.65). All demographic information was found to not be significantly different between groups with and without adenomyosis, including: race (p = 0.75), ethnicity (p = 0.52), body mass index (BMI) (p = 0.89). Investigation of socioeconomic factors, education level (p = 0.20), income (p = 0.43), and employment status (p = 0.65) showed no significant differences. Medical history also revealed no significant differences: menopausal status (p = 0.62), previous dilation and curettage (p = 0.80), co-occurring conditions of endometriosis and fibrosis (p = 0.31 and p = 0.94, respectively), parity (p = 0.09), heaviness of periods (p = 0.61), history of chronic pelvic pain (p = 0.83), history of endometriosis (p = 0.11), history of polycystic ovary syndrome (PCOS) (p = 0.50). For those patients that provided contraceptive data, there was no significant difference found between hormonal (p = 0.24) or non-hormonal (p = 0.99) contraceptive use between the groups. The only significant difference found was use of hormone IUD, with the no adenomyosis group more likely to have used it within the past 6 months (p = 0.02), however frequency of use was small (n = 8). See Table S1 for more information on contraceptive use within the two patient groups.

Immune protein profiling of cervicovaginal samples

To study immunoproteomic differences between patients with and without adenomyosis, we investigated the levels of 72 soluble proteins in CVL samples, including cytokines, chemokines, growth factors, circulating cancer biomarkers, and immune checkpoint proteins. Hierarchical clustering analysis was not able to correctly predict adenomyosis based on global immunoproteomic profiles (Figure S1). To identify proteins in the CVL samples that were altered in women diagnosed with adenomyosis we performed two-sample t-tests and fold change analysis. This revealed that eight soluble proteins were significantly (p<0.05) different in the adenomyosis group compared to the no adenomyosis group (Figure 1A). Cell surface antigen CA19-9 (p = 0.031) and chemokines GROα (p = 0.021) and IP-10 (p = 0.015) were all statistically significantly downregulated in the adenomyosis group compared to women without adenomyosis (Figure 1B). Significantly upregulated proteins in women with adenomyosis included cell surface antigen CEA (p = 0.042) and cytokines: IL-9 (p = 0.047), IL-13 (p = 0.042), IL-36γ (p = 0.017), and TNFβ (p = 0.038), however these were not significant after FDR correction (Figure 1C). These cytokines, specifically IL-9 and IL-13, play an important role in type II immunity and act as chemoattractants for several immune cells, including mast
Table 1. The association of demographics with adenomyosis

| Demographic Category                  | All (n = 108) | Adenomyosis (n = 46) | No Adenomyosis (n = 62) | p-value |
|---------------------------------------|---------------|----------------------|-------------------------|---------|
| **Age (mean (SD))**                   |               |                      |                         |         |
| All                                   | 45.55 (10.01) | 45.52 (8.92)         | 45.58 (10.83)           | 0.65    |
| **Race (n = 107)**                    |               |                      |                         |         |
| American Indian/Alaskan               | 5 (4.67)      | 2 (4.35)             | 3 (4.92)                | 0.73    |
| White/Caucasian                       | 78 (72.90)    | 34 (73.91)           | 44 (72.13)              |         |
| Black or African American             | 11 (10.28)    | 6 (13.04)            | 5 (8.20)                |         |
| All Other                             | 13 (12.15)    | 4 (8.70)             | 9 (14.75)               |         |
| **Ethnicity (n = 108)**               |               |                      |                         | 0.52    |
| Non-Hispanic                          | 76 (70.37)    | 34 (73.91)           | 42 (67.74)              |         |
| Hispanic                              | 32 (29.32)    | 12 (26.09)           | 20 (32.26)              |         |
| **Education (n = 105)**               |               |                      |                         | 0.20    |
| Less than high school                 | 3 (2.86)      | 0 (0.00)             | 3 (5.08)                |         |
| High school diploma or GED            | 20 (19.05)    | 11 (23.91)           | 9 (15.25)               |         |
| Some college                          | 24 (22.86)    | 14 (30.43)           | 10 (16.95)              |         |
| Association degree or Technical degree| 22 (20.95)    | 8 (17.39)            | 14 (23.73)              |         |
| Bachelor degree                       | 22 (20.95)    | 9 (19.57)            | 13 (22.03)              |         |
| Master/Doctor degree                  | 14 (13.33)    | 4 (8.70)             | 10 (16.95)              |         |
| **Household income pre-tax ($) (n = 99)** |           |                      |                         | 0.43    |
| <10,000                               | 3 (3.03)      | 2 (4.35)             | 2 (4.35)                |         |
| 10,000-25,000                         | 10 (10.10)    | 7 (15.22)            | 7 (15.22)               |         |
| 25,000-50,000                         | 13 (13.13)    | 5 (10.87)            | 5 (10.87)               |         |
| 50,000-75,000                         | 23 (23.23)    | 10 (21.74)           | 10 (21.74)              |         |
| 75,000-100,000                        | 15 (15.15)    | 9 (19.57)            | 6 (11.32)               |         |
| >100,000                              | 24 (24.24)    | 8 (17.39)            | 16 (30.19)              |         |
| Don’t know/refused                    | 11 (11.11)    | 5 (10.97)            | 6 (11.32)               |         |
| **Employment status (n = 103)**       |               |                      |                         | 0.65    |
| Yes                                   | 75 (72.82)    | 34 (73.91)           | 41 (67.74)              |         |
| No                                    | 28 (27.18)    | 11 (26.09)           | 17 (29.31)              |         |
| **Marital status (n = 108)**          |               |                      |                         | 0.12    |
| Single/Divorced/Widowed               | 41 (37.96)    | 21 (45.65)           | 20 (32.26)              |         |
| Married                               | 60 (55.56)    | 22 (47.83)           | 38 (61.29)              |         |
| Cohabitating                          | 5 (4.63)      | 1 (2.17)             | 4 (6.45)                |         |
| Other                                 | 2 (1.85)      | 2 (4.35)             | 0 (0.00)                |         |
| **Sexual orientation (n = 100)**      |               |                      |                         | 0.99    |
| Heterosexual                          | 93 (93.00)    | 41 (93.18)           | 53 (92.86)              |         |
| Bisexual                              | 2 (2.00)      | 1 (2.27)             | 1 (1.79)                |         |
| Homosexual                            | 5 (5.00)      | 2 (4.55)             | 3 (5.36)                |         |
| **Alcohol use (current) (n = 101)**   |               |                      |                         | 0.72    |
| Yes                                   | 50 (49.50)    | 23 (53.49)           | 27 (46.55)              |         |
| No                                    | 47 (46.53)    | 19 (44.19)           | 28 (48.28)              |         |
| Quit                                  | 4 (3.96)      | 1 (2.33)             | 3 (5.17)                |         |
| **Tobacco use (within last 6 months) (n = 104)** |       |                      |                         | 0.13    |
| Yes                                   | 14 (13.46)    | 7 (15.62)            | 7 (11.86)               |         |
| No                                    | 34 (32.69)    | 18 (40.00)           | 16 (27.12)              |         |
| Never                                 | 47 (45.19)    | 19 (42.22)           | 28 (47.46)              |         |

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Table 1. Continued

|                           | All (n = 108) | Adenomyosis (n = 46) | No Adenomyosis (n = 62) | p-value |
|---------------------------|--------------|----------------------|-------------------------|---------|
| Quit                      |              |                      |                         |         |
|                           | 9 (8.65)     | 1 (2.22)             | 8 (13.56)               |         |
| Douching (n = 94)         |              |                      |                         | 0.99    |
| Yes                       | 15 (15.96)   | 7 (17.07)            | 8 (15.38)               |         |
| No                        | 79 (84.04)   | 34 (82.93)           | 45 (84.91)              |         |
| BMI (mean (SD) (n = 108)) | 30.63 (7.55) | 30.36 (6.58)         | 30.83 (8.23)            | 0.89    |
| BMI (n = 108)             |              |                      |                         | 0.12    |
| <25                       | 23 (21.30)   | 6 (13.04)            | 17 (27.42)              |         |
| 25–29                     | 38 (35.19)   | 21 (45.65)           | 17 (27.42)              |         |
| 30–34                     | 19 (17.59)   | 9 (19.57)            | 10 (16.13)              |         |
| ≥35                       | 28 (25.93)   | 10 (21.74)           | 18 (29.03)              |         |
| Menopausal status (n = 108) |            |                      |                         | 0.62    |
| Pre                       | 89 (82.41)   | 39 (84.65)           | 50 (80.65)              |         |
| Post                      | 19 (17.59)   | 7 (15.22)            | 12 (19.35)              |         |
| Previous dilation and curettage |        |                      |                         | 0.80    |
| Yes                       | 19 (17.59)   | 9 (19.57)            | 10 (16.13)              |         |
| No                        | 89 (82.43)   | 37 (80.43)           | 52 (83.87)              |         |
| Co-occurring conditions (n = 108) |    |                      |                         | 0.31    |
| Endometriosis             | 21 (19.44)   | 11 (23.09)           | 10 (16.13)              |         |
| Fibroids                  | 70 (64.81)   | 30 (65.22)           | 40 (64.52)              | 0.94    |
| Parity (n = 107)          |              |                      |                         | 0.09    |
| 0                         | 22 (20.56)   | 7 (15.22)            | 15 (24.59)              |         |
| 1                         | 9 (8.41)     | 2 (4.35)             | 7 (11.48)               |         |
| 2                         | 23 (21.50)   | 9 (19.57)            | 14 (22.95)              |         |
| 3                         | 27 (25.23)   | 11 (23.91)           | 16 (26.23)              |         |
| 4+                        | 26 (24.30)   | 17 (36.96)           | 9 (14.75)               |         |
| Heaviness of periods (n = 94) |            |                      |                         | 0.61    |
| Light                     | 5 (5.32)     | 3 (6.82)             | 2 (4.00)                |         |
| Moderate                  | 21 (22.34)   | 8 (18.18)            | 13 (26.00)              |         |
| Heavy                     | 68 (72.34)   | 33 (75.00)           | 35 (70.00)              |         |
| Chronic pelvic pain history (n = 91) | |                      |                         | 0.83    |
| Yes                       | 50 (54.95)   | 22 (56.41)           | 28 (53.85)              |         |
| No                        | 41 (45.05)   | 17 (43.59)           | 24 (46.15)              |         |
| Endometriosis history (n = 95) |            |                      |                         | 0.11    |
| Yes                       | 28 (29.47)   | 16 (39.02)           | 12 (22.22)              |         |
| No                        | 67 (70.53)   | 25 (60.98)           | 42 (77.78)              |         |
| PCOS history (n = 88)     |              |                      |                         | 0.50    |
| Yes                       | 10 (11.36)   | 3 (7.69)             | 7 (14.29)               |         |
| No                        | 78 (88.64)   | 36 (92.31)           | 42 (85.71)              |         |
| Diabetes (n = 108)        |              |                      |                         | 0.15    |
| Yes                       | 22 (20.37)   | 6 (13.04)            | 16 (25.81)              |         |
| No                        | 86 (79.63)   | 40 (86.96)           | 46 (74.19)              |         |
| Hypertension (n = 108)    |              |                      |                         | 0.99    |
| Yes                       | 25 (23.15)   | 11 (23.91)           | 14 (22.58)              |         |
| No                        | 83 (76.85)   | 35 (76.09)           | 48 (77.42)              |         |
| Antibiotics (use within 3 months (n = 95)) | |                      |                         | 0.23    |

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cells and type 2 innate lymphoid cells (ILC2). In contrast, pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, MIP-1β, RANTES, and TNFα were not significantly increased (Figure S2). Levels of CA125 and HE4 were not significantly different between the adenomyosis and the no adenomyosis groups (p = 0.117 and p = 0.09, respectively) (Table S2). Overall, only a few proteins were altered in the CVL samples, mostly immunoregulatory and those involved in type II immune responses.

Global metabolomic profiling of cervicovaginal samples

Next, we used liquid chromatography and mass spectroscopy to determine the metabolic profiles of the CVLs collected from women with benign conditions and identify metabolic differences that may be present between women with and without adenomyosis. Global metabolic analysis detected 912 metabolites, 784 fully characterized compounds and 128 partially characterisable or uncharacterized, within the CVL samples (Figure S3). Global metabolic profiles of patients with adenomyosis and no adenomyosis were created by the data reduction method partial least square-discriminant analysis (PLS-DA). To construct the PLS-DA, two components were used, accounting for 11.6 and 11.1% of the variance of the data. Slight separation with some overlap in overall metabolic profiles was observed between the adenomyosis and no adenomyosis groups (Figure 2A).

The 912 detected metabolites included amino acids (n = 206, 23%), carbohydrates (n = 34, 4%), cofactors and vitamins (n = 32, 4%), energy (n = 12, 1%), lipids (n = 228, 25%), nucleotides (n = 67, 7%), peptides (n = 40, 4%), xenobiotics (n = 165, 18%), partially characterized metabolites (n = 9, 1%) and uncharacterized metabolites (n = 119, 13%). Analysis of superpathway distribution among the metabolites detected revealed a distribution across all superpathways – with amino acids accounting for only 23% of all metabolites. Next, we performed two sample t-tests to determine which metabolites differed significantly between patients with and without adenomyosis, and fold change analysis to identify metabolites that were up/downregulated. The threshold for fold-change analysis was set at 2.0 or greater to be classified as ‘altered’. A large proportion of significantly (p<0.05) altered metabolites were amino acids (n = 43) accounting for more than half of those metabolites (52%) that were altered in adenomyosis compared to no adenomyosis. Furthermore, we performed false-discovery rate (FDR) correction of 10 and 5%, this amino acid signature remained dominant, with amino acids accounting for 56% (n = 22) and 69% (n = 9), respectively (Figure 2B). Fold change analysis and t-tests were combined to produce volcano plots that revealed 82 metabolites that were significantly altered (p<0.05 and fold change>2.0) in adenomyosis patients compared to no adenomyosis, with 1 metabolite being downregulated and 81 upregulated (Figure 2C and Table S3). Statistical analysis with FDR-correction of 10% resulted in 39 significantly upregulated metabolites in adenomyosis (Figure 2D).

Supervised hierarchical clustering analysis (HCA) was performed to depict levels of metabolites within individual samples and identify whether a clear clustering pattern emerged between patients with adenomyosis and patients without adenomyosis. HCA of the top 25 significant metabolites produced a heatmap with a distinct signature, that reflected an enriched metabolic pattern, for adenomyosis compared to no adenomyosis (Figure 2E).

| Table 1. Continued |
|---------------------|-----------------|-----------------|-----------------|---|
|                     | All (n = 108)   | Adenomyosis (n = 46) | No Adenomyosis (n = 62) | p-value |
| Yes                 | 23 (24.21)      | 13 (30.95)       | 10 (18.87)        |         |
| No                  | 72 (75.79)      | 29 (69.05)       | 43 (81.13)        |         |

Patients demographics show no significant difference in demographic, socioeconomic or medical history between adenomyosis and no adenomyosis patients. Values are n (%) unless stated as mean (SD). P-values were calculated using Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables.
N6-acetyllysine (p < 0.0001, q = 0.018), N-formylmethionine (p = 0.0001 and q = 0.028), argininate (p = 0.0002 and q = 0.029), and pipercolate (p = 0.0004 and q = 0.029) were some of the significantly upregulated amino acids in adenomyosis that contributed to the amino acid signatures. These amino acids were selected as they had the most significant p-values, were still significant after FDR-correction of 5% and were detected in at least 85% of adenomyosis patients (Figure 2F). Within the lipid superpathway, 2-hydroxyadipate (p = 0.0003 and q = 0.029) was the only lipid that was significantly upregulated, remained significant after FDR-correction of 5%, and was detected in at least 85% of adenomyosis patients (Figure 2F). To summarise, our analyses show that adenomyosis is distinguished by a unique metabolic signature compared to patients with other benign conditions, particularly by an accumulation of amino acids.

Metabolite enrichment analysis

Next, we performed an enrichment analysis to identify the metabolic pathways that were likely to be altered in patients with adenomyosis compared to patients without adenomyosis based on relative level of metabolites within each group. This revealed that 32 metabolic pathways were significantly (p < 0.05) enriched in adenomyosis patients compared to patients without adenomyosis, the figure shows the top 25 significant pathways (Figure 3). These pathways were associated with nucleotide (n = 1), lipid (n = 6), amino acid (n = 17), or energy metabolism (n = 3), and some were not characterized to a particular superpathway (n = 5). The nucleotide pathway pyrimidine metabolism (p < 0.0001), the lipid pathway carnitine synthesis (p < 0.0001), and the amino acid pathways histidine metabolism (p < 0.0001), and tryptophan metabolism
were among the most significantly enriched (Figures 3, 4A, 4B, and 4C). Scatterplots are shown that represent key metabolites from the pathways; these were the most significant (p<0.05, FC>2.0) and FDR-corrected significantly altered metabolites (q<0.1 and q<0.05) (Figure 4B). From histidine metabolism, key metabolites were N6-acetyllysine (p=0.0001 and q=0.014), N-formylmethionine, argininate, pipecolate, and 2-hydroxyadipate (Figure 4C). For carnitine synthesis, key metabolites were N6, N6, N6-trimethyllysine (p<0.0001 and q<0.01), succinate (p=0.0024 and q=0.069), and deoxycarnitine (p=0.0049 and q=0.088) (Figure 4B). From histidine metabolism, 4-imidazoleacetate (p=0.0025 and q=0.0685), formiminoglutamate (p=0.0051 and q=0.0884), and histamine (p=0.0328 and q=0.2030) were key metabolites; they remained significant after FDR-correction to 10% (q<0.1), except histamine, and were detected in at least 70% of adenomyosis patients (Figure 4C). In total, enrichment analysis revealed that a number of different pathways are enriched within the adenomyosis patients, with the majority of these belonging to the amino acid superpathway. Pyrimidine metabolism, carnitine synthesis, and histidine metabolism were the most significantly enriched pathways.

DISCUSSION
In this study, we investigated the immune protein and metabolic profiles of CVLs from women with benign gynecologic conditions, grouped according to presence (n = 46) or absence (n = 62) of adenomyotic lesions...
and we identified unique immunometabolic signatures associated with adenomyosis. The evaluation of immunometabolic profiles associated with adenomyosis can help us to better understand this enigmatic condition, as well as identify potential biomarker candidates for diagnostic development. Our previous research has shown that non-invasive CVL sampling coupled with immunometabolomic analysis is successful in allowing us to better understand the pathophysiology of conditions such as HPV, cervical dysplasia, and cancer.22–26

To our knowledge, this is the first study investigating soluble proteins present in CVL samples from patients with and without adenomyosis. Out of 72 tested proteins, we identified eight potential protein biomarkers associated with adenomyosis. This included downregulation of two chemokines, IP-10 and GROa. IP-10 is a pro-inflammatory chemokine which has particular involvement in induction of chemotaxis and apoptosis.27,28 GROa is an pro-oncogenic chemokine that plays a role in immune cell trafficking and regulation,29 and has been shown to drive metastatic growth in cancer.30 The downregulation of these two proteins elucidates that there is a state of immune dysregulation that may allow the excess cellular proliferation within the myometrium associated with adenomyosis.

We also found downregulation of CA19-9, a mucin that has been heavily investigated as a biomarker for various cancers.31–33 There is no existing research that adequately explores the levels of CA19-9 in adenomyosis patients. One case study found CA19-9 to be elevated in the serum of one adenomyosis patient.34

Figure 3. Enrichment analysis
Enrichment analysis revealed that 32 pathways were significantly enriched in adenomyosis vs no adenomyosis. (A) Enrichment analysis of adenomyosis patients vs no adenomyosis patients revealed that 32 pathways were significantly (p<0.05) enriched – top 25 pathways are shown here color coded by superpathway and p-value.
Figure 4. Pathway analysis

Pyrimidine metabolism, carnitine synthesis, and histidine metabolism were among the top significantly enriched pathways.

(A) Diagram demonstrating a simplified version of the pyrimidine metabolism pathway and the detection of each metabolite in our analyses. Scatterplots of 3 of the significantly altered metabolites in adenomyosis that demonstrate the enrichment of the pyrimidine metabolism pathway (5,6-dihydrothymine, thymine, N-carbamoylaspartate) color coded by superpathway. Line represents the mean. P- and q-values are shown.

(B) Carnitine synthesis pathway

(C) Histidine metabolism pathway
studies have shown that dysregulation of these immune responses can lead to fibrotic tissue growth.39 an immunoregulatory role by acting as a chemoattractant for neutrophils and other immune cells.44 We have previously found IL-36 reduced in tissue inflammation.39 In addition, IL-9 and IL-13 are produced by type 2 innate lymphoid cells (ILC2) which are also cells involved in the type II immune response.40 ILC2s require IL-9 to increase their promotion of inflammation and cellular proliferation.46 TNF was studied in endometriosis and was proposed as a factor for causing disruption to immune responses by tumor cells while protecting normal cells.47

These results show that CVLs are a non-invasive method that can be utilized to quantify immune proteins with potential diagnostic value. However, the proteins investigated were not sensitive or specific enough alone to stratify patients with and without adenomyosis. Yet, these results show that future investigations in validation cohorts is warranted; combining protein markers in combination with each other or metabolites could lead to a robust diagnostic tool.

Carcinoembryonic antigen (CEA) is another cell-membrane antigen that is overexpressed in various malignancies including mucinous ovarian carcinoma.35 Excess expression of CEA may be involved in reducing local immune response through inhibition of various immune cells.36 Our findings reveal that CEA was increased in cervicovaginal samples from patients with adenomyosis patients, suggesting that it may have the same role within adenomyotic lesions for dampening immune responses. Another study investigating levels of CEA in cervicovaginal fluids from patients with cervical condylomas and cervical intraepithelial neoplasia, found that CEA levels measured locally were more indicative than in serum, and were significantly different from healthy controls.37 Although this provides evidence that CEA is a locally detected biomarker in women with adenomyosis, it may not have great clinical value as it is not specific for only adenomyosis.

Several cytokines were also elevated in the samples from adenomyosis patients compared to patients without adenomyosis; this included IL-9 and IL-13. These cytokines are involved in promoting a type II immune response and act as chemoattractants for mast cells,38 leading to the stimulation of tissue repairs and reduction in tissue inflammation.39 In addition, IL-9 and IL-13 are produced by type 2 innate lymphoid cells (ILC2) which are also cells involved in the type II immune response.40 ILC2s require IL-9 to increase their survival, and to amplify the ILC2 functions within type II immunity, particularly tissue repair.41 Previous studies have shown that dysregulation of these immune responses can lead to fibrotic tissue growth.39 This mechanism of altered tissue repair may be occurring in adenomyosis resulting in uncontrolled tissue growth/fibrosis that promotes the formation of adenomyotic lesions.42

Furthermore, IL-36γ was increased, which conversely is associated with chronic inflammation.43 It also has an immunoregulatory role by acting as a chemoattractant for neutrophils and other immune cells.44 We have previously found IL-36γ to be elevated in cervicovaginal samples from patients with invasive cervical carcinoma45 and bacterial vaginosis.46 Finally, TNFβ was upregulated. This cytokine has previously been studied in endometriosis and was proposed as a factor for causing disruption to immune responses by promotion of inflammation and cellular proliferation.47 TNFβ also has the ability to induce apoptosis within tumor cells while protecting normal cells.48

The levels of CA125 and HE4, both previously investigated as potential biomarkers for adenomyosis, were not significantly different within the CVL samples in our cohort. This highlights the difference that can be observed in detection of these proteins systemically vs. locally. Although minimally invasive blood draws enable measuring these biomarkers in the serum, previous research has showed that these targets do not have high predictive accuracy for adenomyosis systemically.48 These markers are not discriminatory for adenomyosis when measured in CVL samples.

In total, alterations in cervicovaginal protein levels in adenomyosis patients reflect the dysregulation of immune responses. This is induced by attraction of mast cells and induction of ILC2s that promote type II immune responses, leading to the imbalance of tissue repair and inflammatory responses, allowing a state of excess proliferation and tissue growth to occur leading to adenomyotic lesion development within the myometrium.

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In total, alterations in cervicovaginal protein levels in adenomyosis patients reflect the dysregulation of immune responses. This is induced by attraction of mast cells and induction of ILC2s that promote type II immune responses, leading to the imbalance of tissue repair and inflammatory responses, allowing a state of excess proliferation and tissue growth to occur leading to adenomyotic lesion development within the myometrium.

The levels of CA125 and HE4, both previously investigated as potential biomarkers for adenomyosis, were not significantly different within the CVL samples in our cohort. This highlights the difference that can be observed in detection of these proteins systemically vs. locally. Although minimally invasive blood draws enable measuring these biomarkers in the serum, previous research has showed that these targets do not have high predictive accuracy for adenomyosis systemically.48 These markers are not discriminatory for adenomyosis when measured in CVL samples.
Studies assessing the metabolic profiles of adenomyosis patients are also limited with only two small pilot reports published to date. One study used serum samples to investigate systemic metabolic alterations,20 and the other investigated myometrial tissue samples for a local metabolic approach.21 To our knowledge, we are the first to investigate metabolites associated with adenomyosis in CVL samples, which, because of the anatomical continuity of the female reproductive tract, allows successful investigation of the local microenvironment non-invasively.

Our results reveal significant upregulation of metabolites in adenomyosis patients, with a large proportion of these being amino acids, revealing a robust amino acid signature associated with the condition. Previous research has linked amino acid signatures to cancer. Wang et al. showed that amino acid profiles from serum samples distinguished epithelial ovarian cancer patients from healthy controls.49 Song et al., also demonstrated that the metabolic results from myometrial investigation in adenomyosis led to the discovery of significantly altered amino acids and a signature for adenomyosis,21 which is in accordance with our findings in CVL. Compared to previous reports, our study resulted in a greater detection of metabolites (n = 912) and greater number of upregulated amino acids identified, likely because of a more advanced metabolomics platform being utilized. Dysregulation of metabolism has been widely recognized as a hallmark of cancer.50 The uptake and metabolism of amino acids are aberrantly upregulated in many malignancies that display a greater need for amino acids.51 Amino acids facilitate survival and proliferation of cancer cells under genotoxic, oxidative, and nutritional stress.51 This suggests that the hallmarks shared by adenomyosis and cancer, particularly excess cellular proliferation, may be governed by upregulation of amino acids, creating a metabolic signature that can be detected both locally and systemically.

We identified pathways, which may be dysregulated in adenomyosis and therefore provide insights into the pathophysiology of this complex condition. The most significantly enriched pathway in adenomyosis was pyrimidine metabolism. Pyrimidine metabolism is a nucleotide pathway associated with synthesis of nucleic acids,52 a process upregulated during cellular proliferation. A study utilizing a mouse model of adenomyosis found that reduction in enzymatic activity related to the pyrimidine metabolism pathway, which decreases DNA synthesis and reduced incidence of adenomyosis in mice.53 This preclinical report compliments our clinical findings and strongly supports that upregulation of pyrimidine metabolism is a key metabolic signature of adenomyosis.

In addition, another enriched pathway was carnitine synthesis, which is essential for transportation of fatty acids into the mitochondria for fatty acid oxidation.54 Previous research has linked fatty acid oxidation to the development of various cancers, via increased ATP production driving tumor growth.55 Similar to our study, both pyrimidine metabolism and carnitine synthesis were highlighted as pathways that may play pivotal roles in adenomyosis in a previous study utilizing myometrial tissues,21 thus supporting the cervicovaginal signatures identified in our study.

Finally, we also identified histidine metabolism as a significantly enriched pathway within adenomyosis. Histamine synthesis from histidine can occur in mast cells,56 which our immunoproteomic results show are likely recruited to adenomyotic lesions because of IL-9 and IL-13 production. Previous studies have shown that histamine produced by mast cells is linked to uterine contraction.57 Excess release of histamine is also known to cause histamine intolerance within the female reproductive organs, and, in consequence, a key symptom of adenomyosis–dysmenorrhea,58 likely because of uterine contractility.

Herein, we have tested the largest number of protein and metabolic signatures in patients with adenomyosis to date, by utilising non-invasive local CVL sampling. Compared to other studies, we examined a relatively large cohort comprising 108 benign hysterectomy patients, 46 with adenomyosis and 62 without adenomyosis. Although adenomyosis is a condition highly co-occurring with other benign disorders, there were no significant differences in the co-occurrence of fibroids or endometriosis among the groups; nor any significant differences between demographics, socioeconomic background, or medical history (except hormone IUD use, p = 0.02). These factors allowed us to identify a robust, unique signature associated with adenomyosis that warrants further research.

In summary, through immunometabolic profiling of CVL samples, we identified key signatures associated with adenomyosis. The immunoproteomic data was not able to stratify patient groups, however we observed significant increases in cytokines and growth factors related to type II immunity and other
immunoregulatory processes and corresponding metabolic pathways, thereby providing pathophysiological insights into adenomyosis (see graphical abstract). The metabolites and identified metabolic pathways were predictive of disease and have potential diagnostic value. Overall, the immunometabolic pattern validated a novel role for mast cells, ILC2s, proliferation, and the symptomology of adenomyosis. The immunometabolic pattern in CVL samples reflected pathophysiological changes in the upper female reproductive tract, thereby demonstrating the utility of CVL for detection of adenomyosis.

Limitations of study
Although we had a relatively large cohort size for a preliminary study, further research in large and diverse cohorts is required to validate our findings and improve generalisability. Another limitation of our study is the overlap between co-occurring benign gynecologic conditions which influences both our exposed group with adenomyosis and our comparison group; however, we did not observe significant differences in these conditions between our groups. Because of the high prevalence of other benign gynecologic conditions co-occurring with adenomyosis it is not feasible to investigate an exposure group that only has adenomyosis. These other gynecologic conditions may modify the outcomes we investigated. However, our study population is clinically representative of the patients who would be receiving an adenomyosis diagnosis and therefore, appropriate for this analysis. Future analysis that considers other pathologies that may alter the immunometabolic microenvironment are required in order to confirm the signature identified is diagnostic for adenomyosis. Our study also had several strengths, including the global untargeted investigation of metabolites. We detected 912 metabolites, with 784 of those being of known identity and 128 uncharacterized or partially characterized molecules. There is potential for further investigation of the uncharacterized metabolites in the future as they become characterized. To our knowledge this is the largest study to date resulting in the largest metabolomics analysis on adenomyosis.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethical approval
  - Patient recruitment
  - Sample collection
- METHOD DETAILS
  - Soluble proteins quantification
  - Soluble metabolites quantification
  - Partial least squares-discriminant analysis
  - Volcano plot analysis
  - Hierarchical clustering analysis
  - Enrichment analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105508.

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AUTHOR CONTRIBUTIONS

M.M.H.-K supervised the study, led the overall direction, coordinated metabolomics analysis and funding acquisition. J.M. and N.D.M. participated in patient recruitment, sample, and data collection. P.Ł. processed the samples and performed the soluble immune protein experiments. H.C. and D.J.R. performed the statistical analyses. G.M.L., P.Ł., and M.M.H.-K completed literature searches. G.M.L., P.Ł., H.C., D.J.R. and M.M.H.-K analyzed and interpreted the data. G.M.L., P.Ł. and M.M.H.-K drafted and created the visualisation of the data for the paper. All authors critically reviewed and approved the final version of the paper.

DECLARATION OF INTERESTS

M.M.H.-K is a paid consultant for Freya Biosciences. None of this work related to, was shared with, or was licensed to this company or any other commercial entity. G.M.L., P.Ł., D.J.R., J.M., N.D.M., and L.V.F. declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Human cervicovaginal lavage samples | Banner University Medical Center Phoenix Dignity Health Chandler Medical Center | N/A |
| **Chemicals, peptides and recombinant proteins** | | |
| 0.9% saline solution | Teknova, Hollister, CA | Cat#S0699 |
| **Critical commercial assays** | | |
| Milliplex MAP Magnetic Bead Immunoassay: Human Cytokine Chemokine Panel 1 | Millipore, Billerica, MA | Cat# HCYTOMAG-60K |
| Milliplex MAP Magnetic Bead Immunoassay: Human Circulating Cancer Biomarker Panel 1 | Millipore, Billerica, MA | Cat# HCCBP1MAG8-58K |
| Milliplex MAP Magnetic Bead Immunoassay: Human Immuno-Oncology Checkpoint Protein Panel 1 | Millipore, Billerica, MA | Cat# HCKP1-11K |
| Human IL-36γ ELISA kit | RayBiotech, Norcross, GA | Cat#ELH-IL1F9-1 |
| **Softwares and algorithms** | | |
| Bio-Plex Manager 5.0 software | Bio-Rad, Hercules, CA | bio-rad.com |
| **Other** | | |
| Bio-Plex 200 instrument | Bio-Rad, Hercules, CA | Cat#171000201 |
| Metabolon, Inc (global metabolomics platform) | Metabolon, Inc, Durham, NC | metabolon.com |
| MetaboAnalyst 5.0 | MetaboAnalyst | metaboanalyst.ca |

RESOURCE AVAILABILITY

Lead contact
Further information and requests should be directed to and will be fulfilled by the lead contact, Dr Herbst-Kralovetz (mherbst1@arizona.edu).

Materials availability
This study did not generate any new unique reagents.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request. De-identified patient data for soluble immune proteins and significantly altered metabolites is included in the supplemental tables. This study does not report any original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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

Patient recruitment
One hundred and eight participants undergoing hysterectomy for benign conditions were recruited at two clinical sites in the Phoenix (AZ, USA) metropolitan area: Banner University Medical Center – Phoenix and Dignity Health Chandler Medical Center. Histopathology of biopsy samples collected from the surgery were used to stratify into two groups: adenomyosis (n = 46) and no adenomyosis (n = 62). Women were
excluded from the study if they were currently menstruating; currently lactating; currently (or within the past 3 months) on antifungals, antivirals, or topical steroids; currently (or within the past 3 months) had vaginal, vulvar, urinary tract, or sexually transmitted infections; used any douching products, vaginal medications or suppositories, feminine deodorant sprays, wipes, or lubricants within the past 48 h; used any depilatory treatments in the genital area in the past 72 h; had any skin condition in the genital area; had sexual intercourse in the past 48 h; were bathing or swimming in the past 4 h; were smoking or consuming nicotine-containing products in the past 2 h; had hepatitis; were HIV-positive. Inclusion criteria included any women, 18 years of age and older, of any race and ethnicity who were undergoing hysterectomy for benign conditions. Demographic, socioeconomic, and medical history data were collected from surveys and/or medical records.

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

METHOD DETAILS

Soluble proteins quantification
Protein concentrations in CVL samples were measured using the Milliplex MAP Magnetic Bead Immunoassays: Human Cytokine Chemokine Panel 1, Human Circulating Cancer Biomarker Panel 1, and Human Immunology Panel 1 (Millipore, Billerica, MA) according to the manufacturer’s protocol. Levels of 71 proteins (AFP, BTLA, CA15-3, CA19-9, CA125, CD27, CD28, CD40, CD80, CD86, CEA, CYFRA21-1, EGF, eotaxin/CCL11, Fli-3L, FGF-2, fractalkine/CX3CL1, G-CSF, GITRL, GROα/CXCL1, GM-CSF, HE4, HGF, HVEM, ICOS, IFNa2, IFNy, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17A, IP-10/CXCL10, LAG3, leptin, MCP-1/ICIL1, MCP-3/CCL7, MDC/CCL22, MIF, MIP-1α/CCL3, MIP-1β/CCL4, ONP, PD-1, PD-L1, PD-L2, PDGF-AA, PDGF-AB/BB, prolactin, PSA total, RANTES/CCL5, SCF, sCD40L, sFas, sFasL, TGF-α, TIM-3, TLR2, TNFα, TNFβ, TRAIL, VEGF) were quantified using a Bio-Plex 200 instrument and Bio-Plex Manager 5.0 software (Bio-Rad, Hercules, CA). Levels of IL-36γ (IL-1F9) were measured in the samples by enzyme-linked immunosorbent assay using Human IL-36γ ELISA kit (RayBiotech, Norcross, GA) in accordance with the manufacturer’s instructions. All samples were analyzed in duplicate. Concentrations were determined using a five-parameter logistic regression curve fit. If the concentrations measured were below the detection limit, the value was substituted with 0.5 of the minimum detectable concentration provided in the manufacturer’s instructions. Data was normalised using the log10 transformation.

Soluble metabolites quantification
Soluble metabolites in the CVL samples were determined using a global metabolomics platform at Metabolon, Inc. (Durham, NC). Samples were prepared using Micro-Lab STAR® system (Hamilton, Reno, NV). Recovery standards were added for quality control purposes. To recover metabolites and remove protein, the samples were precipitated with methanol under shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. Samples were then placed on a TurboVap® (Zymark) to remove the organic solvent. Samples were split into five aliquots, one for each of the analyses and one spare. A pooled matrix was generated by mixing a small volume of each sample to serve as a technical replicate. Extracted water samples were utilised as process blanks and a mix of quality control standards were selected and added for each sample to monitor instrument performance and aid chromatographic alignment. All methods utilised a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionisation (HESI-II) source and Orbitrap mass analyser operated at 35000 mass resolution. The sample extract was dried and then resuspended in solvents compatible with each of the four methods listed below. The resuspension solvents all contained a series of standards at fixed concentrations for chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, optimised for hydrophilic compounds. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid, and 0.1% formic acid. Another aliquot was...
analyzed using acidic positive ion conditions, optimised for hydrophobic compounds. The extract was
gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% perfluoropentanoic
acid, and 0.01% formic acid and was operated at a higher organic content. The third aliquot was analyzed
using basic negative ion optimised conditions using a separate C18 column. The basic extracts were
gradient eluted using methanol and water, and 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot
was analyzed via negative ionisation following elution from a HILIC column (Water UPLC BEH Amide
2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium
formate, pH 10.8. Peak analysis and quality control processing were performed by the Metabolon Labora-
tory Information System for compound identification. Metabolon’s library is able to match compounds to
more than 3300 purified standards. In addition, recurrent unknown entities were also reported. Peaks were
quantified using area-under-the-curve for relative intensity. Data was normalised by registering the
medians of each compound to equal one and normalising each data point proportionately. Data was trans-
formed using the log10 transformation and autoscaled (mean-centred and divided by the standard devia-
tion of each variable). Percentage fill value data was determined by calculating the percentage of samples
that a particular metabolite was detected in for each group (adenomyosis and no adenomyosis).

Partial least squares-discriminant analysis
Partial least squares-discriminant analysis (PLS-DA) was performed using MetaboAnalyst 5.0.59 to visualise
the separation of the two patient groups: adenomyosis (n = 46) and no adenomyosis (n = 62). PLS-DA is a
supervised regression method that aims to plot the greatest separation between groups by finding the
maximum covariance between the data and the assigned group.

Volcano plot analysis
A two-sample t-test was performed with a significance value threshold of 0.05 pvalue. Fold change analysis
compared the absolute value of change between the means of each metabolite/protein between the two
groups. The fold-change analysis utilises the dataprior tod data transformation and scaling. Data from fold
change and t-test analysis were combined to produce volcano plots that depict the significantly up-/down
regulated metabolites and immune proteins in the women with adenomyosis compared to the women
without adenomyosis. The comparison of direction was adenomyosis vs. no adenomyosis and the fold
change threshold was 2.0. The analysis was performed using MetaboAnalyst 5.0.59

Hierarchical clustering analysis
Partially supervised hierarchical clustering analysis was performed on metabolite and immunoprotein data-
sets, individually, using MetaboAnalyst 5.0.59 to produce heatmaps. Metabolites/immune proteins were
autoscaled and then Pearson distance measure and Ward linkage was applied to the metabolites/immune
proteins. Samples were analyzed both with and without clustering; for those analyzed without clustering,
the order of samples remained in the supervised order inputted, which was categorised based on adeno-
myosis status.

Enrichment analysis
Enrichment analysis was completed in MetaboAnalyst 5.0.59 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 metabolic pathways were calculated based on the number of metabolites
detected within a specific pathway relative to number of known metabolites in that pathway. The algorithm
also considered the relative intensity of the metabolites in adenomyosis compared to no adenomyosis.

QUANTIFICATION AND STATISTICAL ANALYSIS
The data was pre-processed by normalisation, log10 transformation and auto-scaled therefore it was
appropriate to measure the statistical differences between the mean intensities of metabolites among
the groups using a t-test. p-values were corrected using the false discovery rate (FDR) method and q-values
have been reported. p-values<0.05 were considered statistically significant. Statistical analyses were per-
formed using MetaboAnalyst 5.0.9 Differences in demographic, socioeconomic and other patient-related
variables between disease groups (adenomyosis vs. no adenomyosis) were tested using Wilcoxon rank-
sum test for continuous variables and Fisher exact test for categorical variables.