ORIGINAL RESEARCH ARTICLE

Comparative analysis of endometrial, vaginal, and gut microbiota in patients with and without adenomyosis

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Abstract

Introduction: Alterations in microbiota composition have been implicated in a variety of human diseases. Patients with adenomyosis present immune dysregulation leading to a persistent chronic inflammatory response. In this context, the hypothesis that alterations in the microbiota may be involved in the pathogenesis of adenomyosis, by affecting the epigenetic, immunologic, and biochemical functions of the host, has recently been postulated. The aim of the present study was to compare the microbiota composition in the vagina, endometrium, and gut of individuals with and without adenomyosis.

Material and Methods: Cross-sectional study including 38 adenomyosis patients and 46 controls, performed between September 2021 and October 2022 in a university hospital-based research center. The diagnosis of adenomyosis was based on sonographic criteria. Fecal, vaginal, and endometrial samples were collected. Study of the microbiota using 16S rRNA gene sequencing.

Results: Patients with adenomyosis exhibited a significant reduction in the gut microbial alpha diversity compared with healthy controls (Chao1 $p = 0.012$, Fisher $p = 0.005$, Observed species $p = 0.005$). Beta-diversity analysis showed significant differences in the compositions of both gut and vaginal microbiota between adenomyosis patients and the control group (Adonis p-value $= 0.001$; $R^2 = 0.03$ and Adonis p-value $= 0.034$; $R^2 = 0.04$ respectively). Specific bacterial taxa were found to be either overrepresented (Rhodospirillales, *Ruminococcus gauvreauii* group, *Ruminococcaceae*, and *Actinomyces)* or underrepresented in the gut and endometrial microbiota of adenomyosis patients compared with controls. Distinct microbiota profiles were identified among patients with internal and external adenomyosis phenotypes.

Conclusions: The study revealed reduced gut microbiota diversity in adenomyosis patients, accompanied by distinct compositions in gut and vaginal microbiota compared

with controls. Overrepresented or underrepresented bacterial taxa were noted in the gut and endometrial microbiota of adenomyosis patients, with variations in microbiota profiles among those with internal and external adenomyosis phenotypes. These findings suggest a potential association between microbiota and adenomyosis, indicating the need for further research to comprehensively understand the implications of these differences.

KEYWORDS
16S rRNA sequencing, adenomyosis, endometrial microbiota, gut microbiota, vaginal microbiota

1 | INTRODUCTION

Adenomyosis is a benign gynecological condition classically defined by the presence of ectopic endometrial tissue within the myometrium.[1] Recent investigations have led to consider adenomyosis as a heterogeneous clinical condition with at least two different phenotypes based on whether the adenomyotic lesions are in the inner myometrium (internal adenomyosis) or more circumscribed in the outer myometrium (external adenomyosis).[2–5]

Although the exact etiopathology of the disease remains controversial, it is known that patients with adenomyosis present immune dysregulation leading to a persistent chronic inflammatory response, sex steroidal hormone aberrations such as local hyperestrogenism and progesterone resistance, as well as dysregulation in neurogenesis and angiogenesis.[6–8] Moreover, adenomyosis often coexists with endometriosis.[3,9–11]

The commensal microbial communities present along different sites of the human body contribute to the regulation of numerous immune and metabolic functions through a host–microbiota symbiotic interaction, such as the maintenance of mucosal structural integrity, protection against pathogens, synthesis and absorption of essential nutrients, development of the innate and adaptive immune system, modulation of host brain function, and regulation of estrogen metabolism by estrogen-metabolizing enzyme production. Alterations in microbiota composition have been implicated in a variety of human diseases including intestinal alterations, autoimmune and metabolic conditions, cancer, neurological disorders, and gynecological pathologies.[12]

In this context, the hypothesis that alterations in the microbiota may be involved in the pathogenesis of adenomyosis, by affecting the epigenetic, immunologic, and biochemical functions of the host, has recently been postulated.[13–17] Nonetheless, to date, there are still relatively few studies that have focused on characterizing the microbiota of the genital tract in women with adenomyosis,[13–16] and to the best of our knowledge, no study has investigated the intestinal microbiota of these patients.

The present study aimed to compare gut, vaginal, and endometrial microbiota composition, and its diversity between women with and without adenomyosis, and assess whether there are significant differences in microbiota profiles between women with external and internal adenomyosis.

Key message

Our findings imply a possible link between gut, endometrial, and vaginal microbiota compositions and adenomyosis. Moreover, we observed variations in microbiota profiles among patients with internal and external adenomyosis phenotypes, suggesting potential avenues for novel diagnostic and therapeutic approaches.

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2 | MATERIAL AND METHODS

This observational cross-sectional study was performed in a tertiary referral center between September 2021 and October 2022. Patient selection and sampling procedures were carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the study participants.

All consecutive patients between the ages of 18 and 45 years old with a sonographic diagnosis of adenomyosis, with or without concomitant endometriosis, were included in the study. The control group included asymptomatic women visited for routine screening or for reproductive counseling with confirmation of normal uterus and adnexa based on sonographic examination. The exclusion criteria were as follows: (1) postmenopausal status, (2) previous diagnosis of autoimmune, inflammatory and/or neoplastic diseases, (3) use of gonadotropin-releasing hormone agonist, vaginal contraceptive ring or intrauterine device during the 3 months before sample collection, (4) use of antibiotic or pre- or probiotics in the 3 months prior to sample collection, (5) presence of myomas or polyps in the ultrasound examination, and (6) current menstruation.

Adenomyosis was diagnosed according to the following criteria established by the Morphological Uterus Sonographic Assessment (MUSA) group[18,19]: presence of myometrial cysts, myometrial hyperechoic islands, echogenic subendometrial lines, and buds (direct criteria), and asymmetrical thickening of uterine walls, fan-shaped shadowing, translesional vascularity, irregular or interrupted junctional zone, and globular uterus (indirect criteria). Adenomyosis was diagnosed in the presence of two or more criteria, with at least one being direct. Internal adenomyosis

was defined when the junctional zone (inner myometrium) was affected with or without involvement of the middle myometrium and without affecting the outer myometrium (subserosal layer). External adenomyosis, on the other hand, was considered when it involved the outer myometrium, with or without involvement of the middle myometrium, without altering the junctional zone. The presence of ovarian and deep endometriosis was also assessed according to the International Deep Endometriosis Analysis (IDEA) group consensus.[20] The following socio-demographic and clinical data were collected from each patient during the medical visit performed following the ultrasound examination: age, ethnicity, body mass index, infertility, nulliparity, and hormonal treatment. Dysmenorrhea and non-cyclic chronic pelvic pain were also assessed using a numerical rating scale, where 0 represented no pain and 10 indicated unbearable pain. Additionally, the presence of heavy menstrual bleeding was evaluated based on subjective patient assessments.[21]

2.1 | Sample collection and processing

Vaginal samples were collected using swabs (Cytobrush Plus GT; Medscan Medical AB) from the posterior vaginal fornix following the insertion of a sterile speculum and before performing any vaginal procedure including pelvic examination or transvaginal ultrasound. Each cytobrush was submerged in 1.0 mL of sodium chloride (NaCl) (9 mg/mL). Subsequently, the vagina and external cervix were swabbed with chlorhexidine. Endometrial samples were then collected using a double-sheathed, sterile pipelle endometrial suction curette that was passed through the cervix to collect an endometrial biopsy, taking care to avoid contact with the vaginal wall and cervix. Biopsies were submerged in 1.0 mL of NaCl (9 mg/mL). Samples were transported to the laboratory on ice and preprocessed within 1 hour. Vaginal fluid and endometrial samples were centrifuged at 3000 g at 4°C for 10 min and pellets were stored at −80°C until being processed.

Additionally, 0.5–1.0 g of fecal samples were collected at home on the same day of the visit by the participants using the DANASTOOL Sample Collection MICROBIOME Kit (Danagen-Bioted, BCN, Spain) and provided to the clinic. After homogenization with phosphate-buffered saline (PBS) buffer, the fecal samples were stored at −80°C.

2.2 | DNA extraction, 16S ribosomal ribonucleic acid (16S rRNA) amplification, and sequencing

DNA from fecal, endometrial, and vaginal samples was extracted using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA) on the Thermo Scientific™ KingFisher™ Flex Purification System and following the Soil Flex protocol according to the manufacturer's instructions. The extracted DNA was quantified using the QuantiFluor ONE dsDNA Dye

(Promega, Madison, WI, USA) on a Quantus Fluorometer (Promega, Madison, WI, USA).

The 16S rRNA V3-V4 region was amplified using previously described primers with Illumina adapter sequences.[22] Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Illumina protocol (Part # 15044223 Rev. A, Illumina, CA, USA). Libraries were diluted to 1.2 nM concentration, pooled in equimolar proportions, and loaded at 5 pM concentrations with 20% 5 pM PhiX on a MiSeq platform (2×300 bp).

2.3 | Sequence processing and statistical analyses

Sequences were processed using QIIME2-2022.2[23] and DADA2 pipeline for quality control, applying the following parameters: −-p-trim-left-f 30, −-p-trim-left-r 0, −-p-trunc-len-f 280, and −-p-trunc-len-r 220. All samples were rarefied at 2000 sequencing depth based on α-diversity rarefaction plots and taxonomy was assigned using the Silva v138.1 database trained on the V3-V4 16S rRNA region with a Naive Bayes classifier. Alpha and beta diversity analyses were performed using *phyloseq* v.1.38.0, *picante* v.1.8.2, and *vegan* v.2.5–7 packages using R v.4.1.2 and Rstudio v.2022.2.3 environments.

For alpha diversity, Chao1, Faith's PD, Fisher, Observed species, Shannon, and Simpson indexes were calculated and compared using the nonparametric Wilcoxon test. Beta diversity was assessed with Non-metric Multidimensional Scaling using Bray–Curtis dissimilarity index matrices. The statistical significance of sample groupings was calculated using the resulting distance matrices and the Adonis nonparametric analysis of variance. $p < 0.05$ was considered statistically significant in both alpha and beta diversity analyses.

Differentiation of microbial abundance at a genus level was assessed by the utilization of linear discriminant analysis effective size (LEfSe)[24] establishing a threshold of 2.0 linear discriminant analysis score and 0.05 α-significance level to report significant taxa.
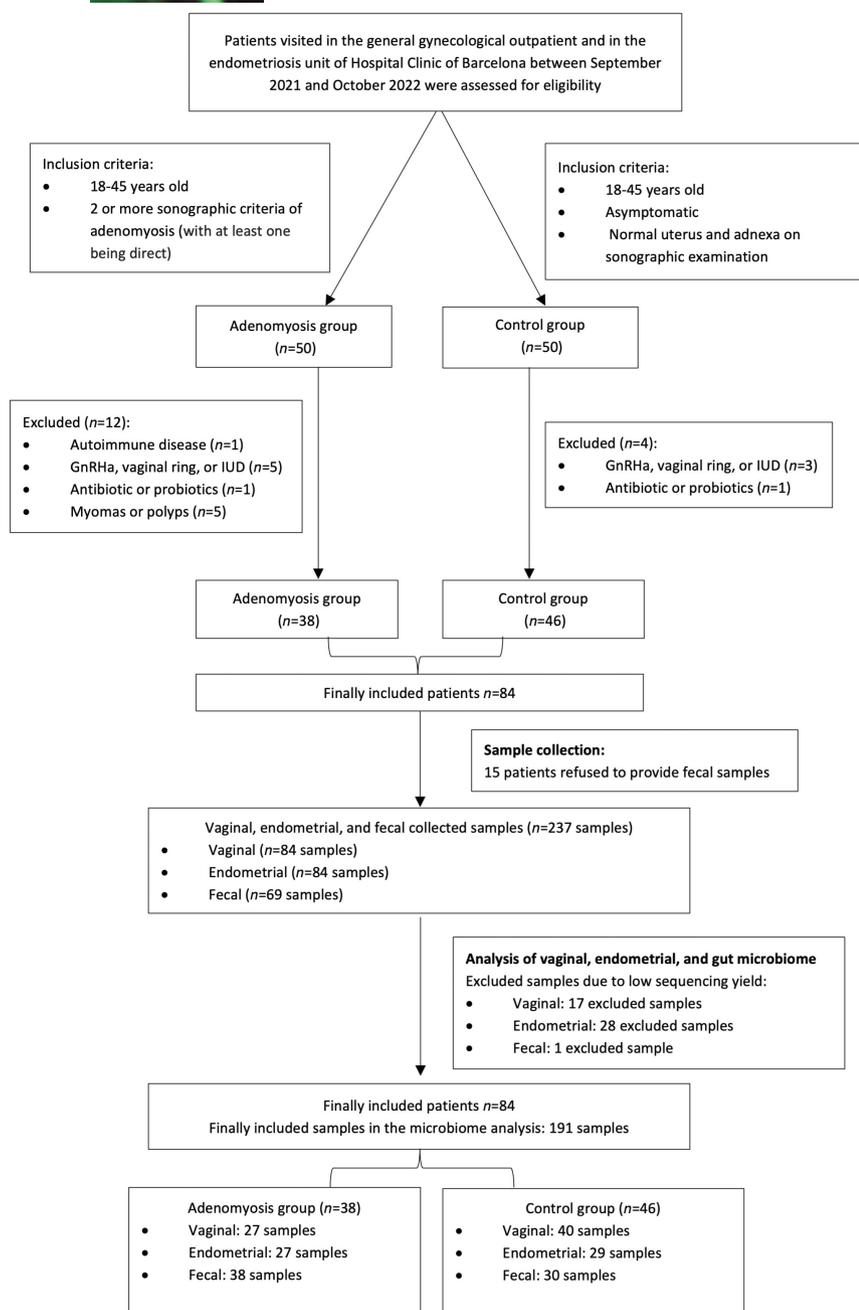
Other statistical analyses were performed with Stata software version 15.1. Continuous variables were analyzed by *t*-tests while categorical variables were analyzed by Fisher's exact test; $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Samples and participant characteristics

A total of 84 women were included in the study, 38 (45.2%) in the adenomyosis group and 46 (54.8%) in the control group (Figure 1). The baseline socio-demographic and clinical characteristics of the women included are shown in Table 1. No significant differences were found among groups regarding age, ethnicity, hormonal treatment, and body mass index, whereas women with

Patients visited in the general gynecological outpatient and in the endometriosis unit of Hospital Clinic of Barcelona between September 2021 and October 2022 were assessed for eligibility

Inclusion criteria:
- 18-45 years old
- 2 or more sonographic criteria of adenomyosis (with at least one being direct)

Inclusion criteria:
- 18-45 years old
- Asymptomatic
- Normal uterus and adnexa on sonographic examination

Adenomyosis group (*n*=50)

Control group (*n*=50)

Excluded (*n*=12):
- Autoimmune disease (*n*=1)
- GnRHa, vaginal ring, or IUD (*n*=5)
- Antibiotic or probiotics (*n*=1)
- Myomas or polyps (*n*=5)

Excluded (*n*=4):
- GnRHa, vaginal ring, or IUD (*n*=3)
- Antibiotic or probiotics (*n*=1)

Adenomyosis group (*n*=38)

Control group (*n*=46)

Finally included patients *n*=84

Sample collection:
15 patients refused to provide fecal samples

Vaginal, endometrial, and fecal collected samples (*n*=237 samples)
- Vaginal (*n*=84 samples)
- Endometrial (*n*=84 samples)
- Fecal (*n*=69 samples)

Analysis of vaginal, endometrial, and gut microbiome
Excluded samples due to low sequencing yield:
- Vaginal: 17 excluded samples
- Endometrial: 28 excluded samples
- Fecal: 1 excluded sample

Finally included patients *n*=84
Finally included samples in the microbiome analysis: 191 samples

Adenomyosis group (*n*=38)
- Vaginal: 27 samples
- Endometrial: 27 samples
- Fecal: 38 samples

Control group (*n*=46)
- Vaginal: 40 samples
- Endometrial: 29 samples
- Fecal: 30 samples

adenomyosis were more frequently nulliparous. With respect to clinical symptoms, patients with adenomyosis presented significantly more dysmenorrhea, non-cyclic chronic pelvic pain, heavy menstrual bleeding, and infertility than healthy controls. Thirty-four (89.5%) of the patients with adenomyosis had associated endometriosis. Table 2 provides details of the sonographic findings on the patients with adenomyosis.

Vaginal and endometrial samples were collected from each participant but only 69 (82.1%) women agreed to deliver fecal samples. Among the 237 samples collected and processed, 46 (19.4%) were excluded due to low sequencing yield (Figure 1; Supporting information Tables S1 and S2).

A total of 10 222 309 raw sequences, with 14 889 median sequences per sample (interquartile range 4321–25 511) were

obtained. According to alpha rarefaction curve estimation, a sequencing depth of 2000 sequences per sample may represent the microbial diversity of each community. Considering all the collected samples, we were able to identify 312 different bacterial genera and 104 different bacterial families.

3.2 | Diversity analysis

Alpha and beta diversity analyses were conducted to assess the differences in the composition of vaginal, endometrial, and gut microbiota in adenomyosis patients and healthy controls.

Alpha diversity comparison between groups showed that the Chao1 ($p = 0.012$), Fisher ($p = 0.005$), and the observed species

TABLE 1 Baseline clinical characteristics of the women included.

	Total $n=84$	Adenomyosis $n=38$	Controls $n=46$	p-value
Age in years, mean (SD)	37.6 (4.9)	38.0 (4.9)	37.1 (4.9)	0.38
BMI in kg/m^2, mean (SD)	24.4 (4.0)	24.4 (4.2)	24.4 (3.9)	0.98
Caucasian, n (%)	72 (85.7)	34 (89.5)	42 (91.3)	0.23
Nulliparity, n (%)	40 (47.6)	27 (71.1)	13 (28.2)	**<0.001**
Hormonal treatment, n (%)	68 (81.0)	31 (81.6)	37 (80.4)	0.56
Pain scores, NRS (0–10), mean (SD)				
Dysmenorrhea	4.5 (3.6)	5.7 (3.9)	3.6 (3.1)	**0.007**
NCCPP	2.1 (2.9)	4.2 (3.1)	0.4 (1.2)	**<0.001**
HMB, n (%)	20 (23.8)	20 (52.6)	0 (0.0)	**<0.001**
Infertility	12 (14.3)	12 (31.6)	0 (0.0)	**<0.001**

Abbreviations: BMI, body mass index; HMB, heavy menstrual bleeding; NCCPP, non-cyclic chronic pelvic pain; NRS, numerical rating scale.

Note: Bold values indicate statistically significant results ($p < 0.05$).

TABLE 2 Sonographic features in the adenomyosis group.

Adenomyosis phenotype, n (%)	
Internal	29 (76.3)
External	9 (23.7)
Number of adenomyosis criteria, n (%)	
2	1 (2.6)
3	5 (13.2)
4	18 (47.4)
5	5 (13.2)
6	6 (15.9)
7	3 (7.9)
Adenomyosis criteria, n (%)	
Myometrial cysts	17 (44.5)
Myometrial hyperechogenic islands	37 (97.4)
Wall asymmetry	19 (50.0)
Fan-shaped shadowing	31 (81.2)
Irregular or interrupted JZ	29 (76.3)
Translesional vascularity	18 (47.4)
Globular uterus	22 (57.9)
Ovarian endometriosis, n (%)	19 (50.0)
Deep endometriosis, n (%)	34 (89.5)
Deep endometriosis location, n (%)	
Torus and/or uterosacral ligaments	34 (89.5)
Rectosigmoid	19 (50.0)
Bladder	2 (5.3)

($p=0.005$) indexes in the fecal samples of the control group were significantly higher than in the adenomyosis group (Figure 2). In contrast, no significant differences in alpha diversity were found between groups regarding vaginal and endometrial microbiota.

Regarding beta diversity, gut and vaginal microbiota of patients with adenomyosis and controls significantly differed (Adonis p-value$=0.001$; $R^2=0.03$ and Adonis p-value$=0.034$; $R^2=0.04$ respectively; Supporting information Figure S1), whereas there were no significant differences between groups regarding the composition of the endometrial microbiota (Adonis p-value$=0.079$; $R^2=0.03$).

3.3 | Relative taxonomy abundance between adenomyosis and control groups

The distribution of the top 20 taxa of vaginal, endometrial, and gut microbiota at the family level is depicted in Figure 3. *Bacteroidaceae* was the most abundant family found in fecal samples in both groups, followed by *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*, and *Oscillospiraceae*. In vaginal and endometrial samples, *Lactobacillaceae* was the most abundant family found in both groups, followed by *Bifidobacteriaceae*. The third most abundant family found in endometrial and vaginal samples of adenomyosis patients was *Streptococcaceae*, being *Atopobiaceae* in the control group.

3.4 | Differential analysis of taxonomy profiles

LEfSe identified differentially abundant bacterial taxa in the gut and endometrial microbiota of patients with adenomyosis and controls (Figure 4). A significant increase in the abundance of the order *Rhodospirillales* and genus *Ruminococcus gauvreauii* group was identified in fecal samples in the adenomyosis group. On the other hand, we found that families *Clostridiaceae*, *Rikenellaceae_RC9_gut_group*, *Peptostreptococcaceae* and *Atopobiaceae* and genus *Bifidobacterium*, *Streptococcus*, *Sutterella*, *Holdemanella*, *Coprococcus*, *Dorea*, *Butyricicoccus*, *Veillonella*, *Clostridium_sensu_stricto_1*, *UBA 1819*, *Acidaminococcus*, *Olsenella*, *Allisonella*, and *Howardella* were significantly more abundant in the control group. In endometrial
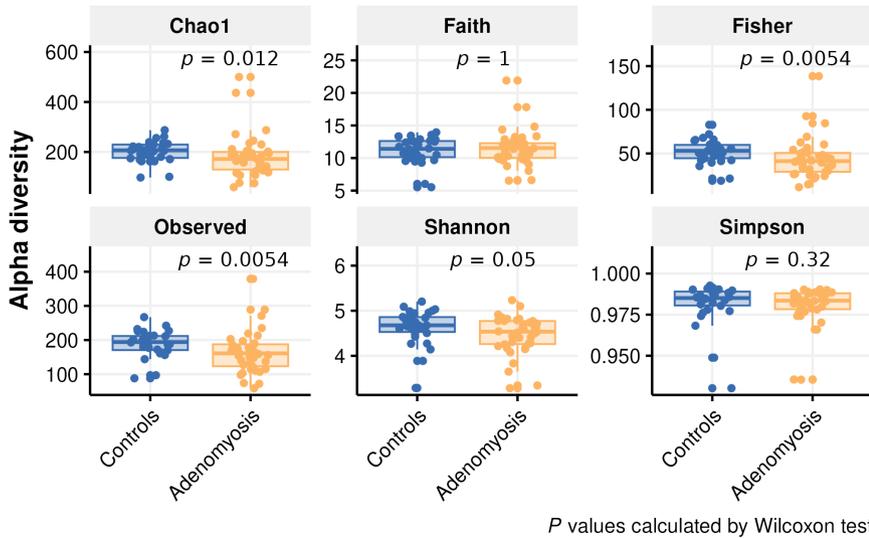
FIGURE 2 Boxplots representing alpha diversity analysis results for fecal samples including Chao1, Faith's PD, Fisher, observed species, Shannon, and Simpson indexes. The Chao1 ($p=0.012$), Fisher ($p=0.005$), and the observed species ($p=0.005$) in the fecal samples of the control group were significantly higher than in the adenomyosis group ($p<0.05$).
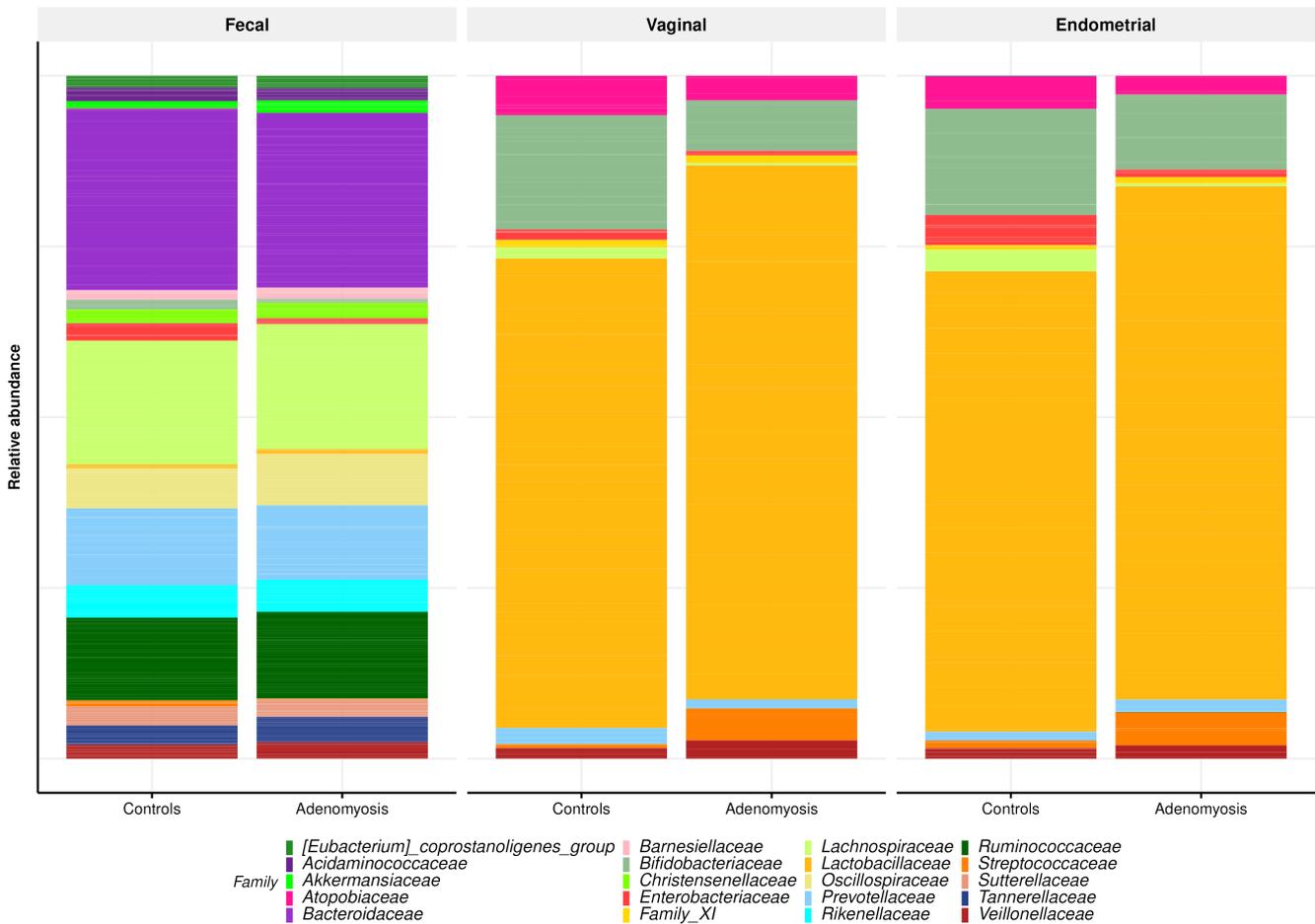
P values calculated by Wilcoxon test

FIGURE 3 Distribution of the top 20 taxa of vaginal, endometrial, and gut microbiota at the family level.

samples, the family *Ruminococcaceae* and genus *Actinomyces* were significantly enriched in the adenomyosis group. Moreover, the family *Enterobacteriaceae* and genus *Enterobacter, Ligilactobacillus, Lacticaseibacillus,* and *Fastidiosipila* were significantly more abundant in the control group. In vaginal samples, no differentially abundant bacterial taxa were found among groups.

(A) Gut microbiota
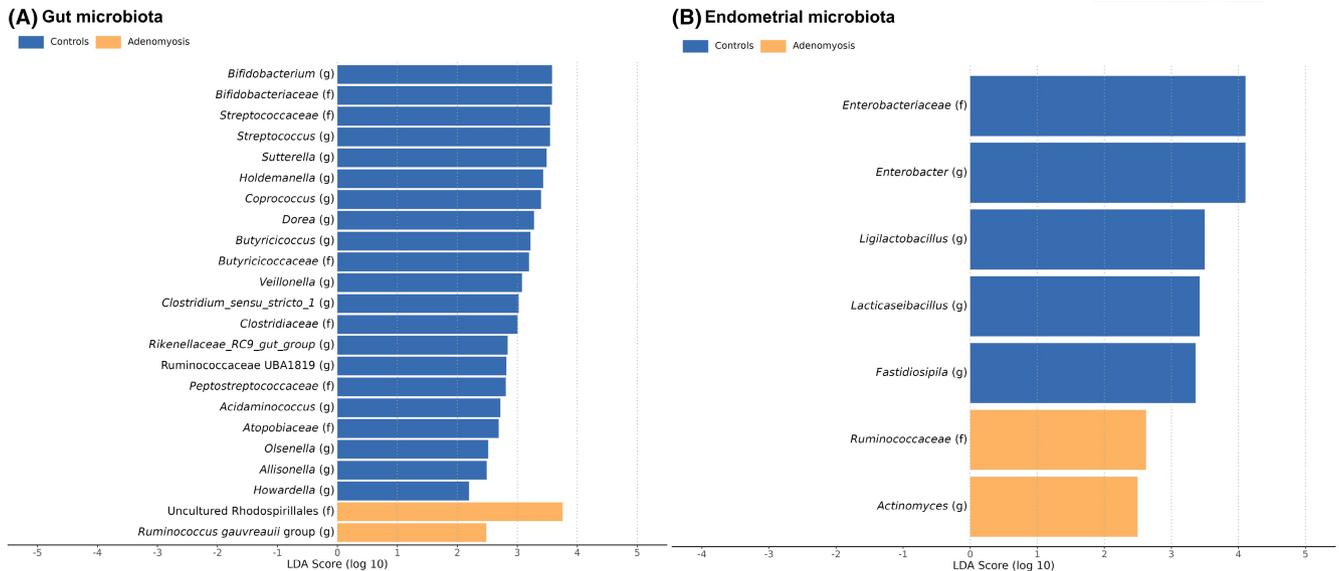
(B) Endometrial microbiota

FIGURE 4 Differentially abundant bacterial taxa in the gut (A) and endometrial (B) microbiota of patients with adenomyosis and controls based on linear discriminant analysis (LDA) effect size technique (LEfSe) analysis.
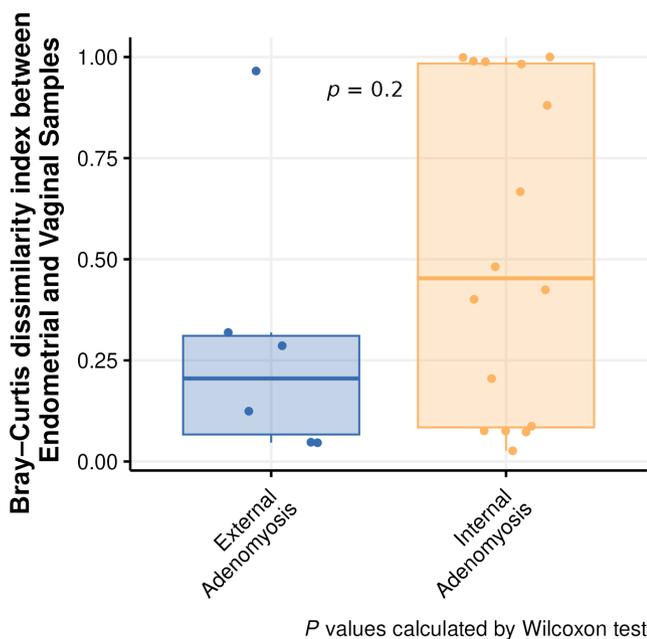

FIGURE 5 Boxplots representing differences in Bray–Curtis dissimilarity index between endometrial and vaginal samples of patients with internal and external adenomyosis.

3.5 | Adenomyosis phenotypes and microbiota

No statistical significance in the alpha and beta-diversity analysis was found between internal and external adenomyosis within the three studied sites (feces, vagina, and endometrium). Nonetheless, the beta-diversity analysis showed that, albeit not statistically significant, there were more differences between the composition of vaginal and endometrial microbiota in patients with internal adenomyosis than in patients with external adenomyosis (Figure 5).

Moreover, the LEfSe analysis identified differentially abundant bacterial taxa in the three studied sites of patients with internal and external adenomyosis as shown in Figure 6.

3.6 | Concomitant endometriosis in patients with adenomyosis and microbiota

In the adenomyosis group, no statistical significance was found in the alpha and beta-diversity analysis between with and without associated ovarian endometriosis, nor between patients with and without associated deep infiltrating endometriosis across the three studied sites (feces, vagina, and endometrium). However, the LEfSe analysis identified differentially abundant bacterial taxa in the fecal samples of patients with adenomyosis and associated rectosigmoid deep infiltrating endometriosis, as shown in Supporting information Figure S2.

4 | DISCUSSION

In this study, we compared the vaginal, endometrial, and gut microbiota composition of patients with adenomyosis, with or without concomitant endometriosis, and healthy controls. The main findings of the present study are: the diversity of gut microbiota was significantly lower in the adenomyosis group (alpha-diversity analysis); both gut and vaginal microbiota compositions significantly differed between adenomyosis patients and controls (beta-diversity analysis); certain bacterial taxa were found to be overrepresented or underrepresented in the gut and endometrial microbiota of adenomyosis patients compared with controls, and finally, some differences in microbiota profiles were identified between patients with internal and external adenomyosis phenotypes.
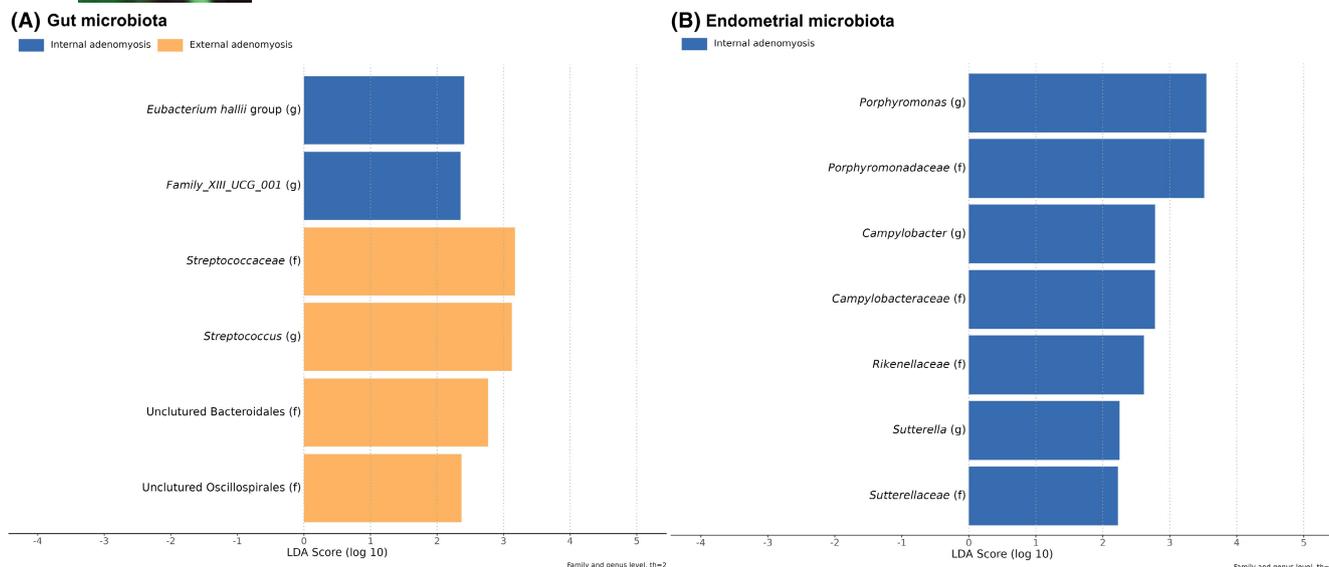
(A) Gut microbiota

■ Internal adenomyosis ■ External adenomyosis

(B) Endometrial microbiota

■ Internal adenomyosis

FIGURE 6 Differentially abundant bacterial taxa in the gut (A) and endometrial (B) microbiota of patients with internal and external adenomyosis based on linear discriminant analysis (LDA) effect size technique (LEfSe) analysis.

It is known that the gut microbiota has the potential to impact the overall human physiology by influencing intestinal permeability, digestive and endocrine metabolism, and immune responses.[25] Eubiotic gut microbiota is characterized by being diverse and balanced and is primarily composed of bacteria dominated by the two phyla *Firmicutes* and *Bacteroidetes* which, when combined, represent 90% of gut microbials in healthy individuals.[26] In recent years, it has been acknowledged that gut dysbiosis, defined by an alteration in gut microbiota composition and reduction in its diversity, is associated with intestinal and extra-intestinal conditions, such as irritable bowel syndrome, inflammatory bowel disease, celiac disease, colorectal cancer, metabolic disorders (obesity and type 2 diabetes), Alzheimer's and Parkinson's diseases and autism spectrum disorders.[27] Moreover, research has demonstrated that the gut microbiota can also have an impact on estrogen levels and estrogen-related diseases.[17,28] To date, some previous studies have investigated the connection between gut microbiota and endometriosis in humans,[29–32] but only one study centered on adenomyosis, based on an animal model, has been published.[17] Therefore, to our knowledge this is the first study that analyzes changes in the intestinal microbiota in patients with adenomyosis, with or without associated endometriosis, compared with healthy controls.

In our study, the diversity of gut microbiota was significantly lower in adenomyosis patients. Moreover, beta-diversity analysis showed that gut microbiota composition differs between adenomyosis patients and controls. The only previous study focused on gut microbiota and adenomyosis was performed in mice and did not find differences regarding alpha and beta diversity between groups. Nonetheless, loss of microbiota diversity is of note the most constant finding of intestinal dysbiosis and has previously been linked to different chronic conditions[33] including endometriosis.[34]

On the other hand, most of the taxonomic groups identified as significantly differing in abundance in fecal samples between the adenomyosis and control groups by LEfSe analysis are known for either their favorable or detrimental effects on intestinal equilibrium. Adenomyosis patients presented a reduction in the genus *Bifidobacterium* in gut microbiota compared with the control group. *Bifidobacterium* is known to have beneficial effects such as short-chain fatty acids (SCFA) production, biological barrier formation, and anti-microbial compound secretion.[35] Interestingly, most of the other depleted bacterial taxa in the adenomyosis group (genus *Coprococcus*, *Dorea*, *Veillonella*, *Butyricicoccus*, *and Allisonella*) are important butyrate producers[36–38] and some have also been found to be reduced in inflammatory bowel disease,[39] Crohn's disease,[40] and endometriosis.[30] Butyrate is a SCFA found in the gut metabolome that serves as a crucial energy source for enterocytes and stimulates the growth of the intestinal epithelium, repairing and fortifying the gut barrier.[41] A decrease in the abundance of taxa responsible for butyrate production may lead to compromised epithelial cell integrity, increased colonic permeability, bacterial infiltration, and local inflammation.[42] On the other hand, patients with adenomyosis exhibited an enrichment of the *Ruminococcus gauverii* group and the *Rhodospirillales* order compared with the control group. *R. gauverii* is a mucolytic bacterium from the *Ruminococcaceae* family which can induce chronic intestinal inflammation and disruption of the gut barrier.[43–46] Moreover, previous studies showed that genus from the *Ruminococcaceae* family are beta-galactosidase and beta-glucuronidase producers.[47,48] The estrobolome has been considered a significant contributor to adenomyosis and endometriosis by dysregulating circulating estrogen levels in women, through gut microbial enzymes involved in their metabolism. Particularly, beta-glucuronidase, beta-galactosidase, and beta-glucosidase, are bacterial hydrolytic enzymes that play a key role in the deconjugation of estrogens from glucuronic acid, leading to their reabsorption in active form into the circulatory system.[28,49] Taxa from the order *Rhodospirillales* are

Gram-negative bacteria and, therefore, are characterized by having lipopolysaccharide (LPS) as the main component of their outer membrane. LPS functions as a powerful endotoxin, and its activation of pattern recognition receptors, such as toll-like receptor 4, plays a crucial role in provoking pro-inflammatory and immune responses, as well as promoting neo-angiogenesis and the secretion of growth factors. All these mechanisms are known to be involved in the pathogenesis of adenomyosis.[8,25,50–52]

Furthermore, it has been demonstrated that the complex interplay among bacteria and the host epithelial and immune cells within the female reproductive tract is crucial for preserving the homeostasis of the reproductive system, and that a shift in their equilibrium can potentially contribute to a wide range of conditions.[28,53–55] Even if healthy women may exhibit diverse vaginal microbial compositions,[56] eubiotic vaginal microbiota is normally associated with low bacterial diversity and the dominance of *Lactobacillus* spp.[57] Despite having been less studied to date, the uterine microbiota in healthy women is characterized by an abundance of genus *Lactobacillus*, genus *Gardnerella* and phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*.[54,58,59] Compared with the vaginal microbiota, the endometrial microbiota presents a significantly lower density—with approximately 10000 times fewer bacteria—but it displays a higher degree of bacterial diversity.[54,60] Some studies have previously examined the microbiota in the female reproductive tract of women with adenomyosis[13–16] revealing differences compared with control groups. These findings suggest a potential link between female reproductive tract dysbiosis and the pathogenesis of adenomyosis.

In terms of vaginal microbiota, we found no significant differences in alpha diversity, but beta diversity analysis revealed that vaginal microbiota composition significantly differed between women with adenomyosis and healthy controls. These findings align with data previously published by Chen et al. in 2020,[16] and Chao et al. in 2021.[15] In their recently published study, Kunaseth et al.[14] found that the richness of vaginal microbiota was significantly higher in patients with adenomyosis compared with healthy controls despite the absence of differences between groups in the beta diversity analysis.

Regarding endometrial microbiota, we found no significant differences in alpha-diversity and beta-diversity analysis among groups. Our findings are not in line with a prior study conducted by Lin et al.,[13] which reported a significant reduction in endometrial microbial richness of patients with adenomyosis compared with healthy controls, along with significant variations in endometrial microbiota composition (beta diversity) between the adenomyosis and control groups. However, our LEfSe analysis showed significantly differential abundances of endometrial bacteria between groups: We found that the *Ruminococcaceae* family and the genus *Actinomyces* were significantly enriched in the adenomyosis group, whereas the genus *Ligilactobacillus*, *Lacticaseibacillus*, and *Fastidiosipila* were significantly more abundant in the control group. In their recent work, Lin et al. also detected an enrichment of *Actinobacteria* (class) in endometrial samples from patients with adenomyosis.[13] Taxa from the *Ruminococcaceae* family are SCFA-producers. As mentioned

before, SCFAs, such as acetate and butyrate, are known to play an anti-inflammatory and protective role in the gut. Interestingly, in the female reproductive tract, SCFAs have been observed to promote dysbiosis and inflammation, especially when there is an associated deficiency in lactate-producer lactobacilli. The increase in SCFAs in the female reproductive tract has been associated with enhanced pro-inflammatory cytokine production through Toll-like receptor activation and reduced neutrophil chemotaxis. Moreover, the reduction in the abundance of the genus *Ligilactobacillus* and *Lacticaseibacillus* among individuals with adenomyosis aligns with prior research findings that have reported lower levels of these lactate-producer bacteria in cases of other pathologic gynecological conditions.[61–63]

We identified differentially abundant bacterial taxa in the gut and endometrial microbiota of patients with internal and external adenomyosis. Moreover, although not statistically significant, it seems that there are more differences between the composition of vaginal and endometrial microbiota in patients with internal adenomyosis than in patients with external adenomyosis. These findings could be associated with the previously suggested theory indicating distinct pathways in the development of internal and external adenomyosis, with internal adenomyosis possibly originating from the endometrium within the uterus, and external adenomyosis arising from ectopic endometrial cells invading from a nearby endometriotic lesion.[3,4,64]

Most of the patients with adenomyosis included in our study had associated endometriosis, which is not unexpected and is consistent with previously published literature.[3,9–11] Although it was not a primary outcome of our study, we evaluated whether there could be differences in the microbiota profiles of patients with adenomyosis due to the presence of concomitant endometriosis. We found no statistical significance in diversity analysis between patients with and without associated ovarian endometriosis, nor between patients with and without associated deep infiltrating endometriosis across the three studied sites. However, the LEfSe analysis identified differentially abundant bacterial taxa in the fecal samples of patients with adenomyosis and associated rectosigmoid deep infiltrating endometriosis. Further studies are warranted to comprehensively investigate the potential implications of these findings.

Our study has several strengths that should be considered: it is the first study describing the composition of the gut, vaginal, and endometrial microbiota of patients with adenomyosis, with or without associated endometriosis; moreover, it is the first study comparing microbiota profiles between internal and external adenomyosis phenotypes. Additionally, this study was performed in a tertiary referral center where the transvaginal ultrasound evaluations were performed by two expert sonographers with more than 10 years of experience and who have previously demonstrated a high diagnostic accuracy with transvaginal ultrasound for determining the presence of adenomyosis.[65,66] Finally, all vaginal and endometrial samples were collected by the same investigator following a strict protocol to avoid contamination and were later processed and analyzed using a blinded approach to ensure

the accuracy of the statistical analyses. On the other hand, we must acknowledge certain limitations. Our study population was selected from the Endometriosis Unit of a tertiary center and, therefore, patient selection bias may be present as the high rate of patients with associated endometriosis has decreased the external validity of our study making our results not applicable to the general population. Moreover, most of the patients included in our study were undergoing hormonal therapy, which is our first-line treatment for adenomyosis and endometriosis. While this could be considered a potential source of bias, there were no significant differences in hormonal treatment intake among the groups ($p = 0.56$). Furthermore, a recent study has shown that the use of hormonal contraceptives is not associated with differences in microbiota composition or diversity.[67] Nevertheless, it would be valuable to investigate the potential impact of hormonal treatment on the microbiota of adenomyosis patients through prospective studies analyzing microbiota composition before and after the initiation of hormonal therapy for each individual patient. Such investigations could shed light on whether hormonal treatments influence the microbiota and whether any alterations in microbiota composition correlate with treatment outcomes or symptom management. Understanding these potential relationships could potentially lead to more tailored and effective treatment approaches. Another limitation of our study is that we used 16S rRNA gene sequencing, which limited the taxonomic resolution. Using alternative methods, such as shotgun metagenomic sequencing, would enable the identification and profiling of a broader range of microorganisms, including fungi, viruses, and microbial genes, thereby providing additional insights into the functional potential of the microbiota, as well as a better resolution for the bacterial taxonomic classification.

5 | CONCLUSION

Our study investigated the composition of the microbiota in the vagina, endometrium, and gut of patients with and without adenomyosis. The results revealed that adenomyosis patients displayed reduced diversity in the gut microbiota. Moreover, significant variations were observed in both gut and vaginal microbiota compositions between adenomyosis patients and the control group, and specific bacterial taxa were either overrepresented or underrepresented in the gut and endometrial microbiota of adenomyosis patients compared with controls. Notably, differences in microbiota profiles were identified between patients with internal and external adenomyosis phenotypes. These findings shed light on the potential role of microbiota in the context of adenomyosis and warrant further research to better understand the implications of these differences.

AUTHOR CONTRIBUTIONS

Marta Valdés-Bango, Meritxell Gracia, Andrea Vergara, Climent Casals-Pascual, and Francisco Carmona planned and designed the study. Marta Valdés-Bango, Meritxell Gracia, Mariona Rius, Maria Ángeles

Martínez-Zamora, Cristina Ros, and Lara Quintas included participants, collected samples, and provided clinical data. Marta Valdés-Bango and Elisa Rubio processed samples. Marta Valdés-Bango, Meritxell Gracia, Elisa Rubio, and Eduard Mension analyzed data. All authors contributed to data interpretation. Marta Valdés-Bango, Meritxell Gracia, Elisa Rubio, and Francisco Carmona contributed to writing the manuscript. All authors contributed to the manuscript revision. All the authors approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT
No conflict of interest to declare.

ETHICS STATEMENT
Patient selection and sampling procedures were carried out in accordance with the Declaration of Helsinki and applicable local regulatory requirements after approval from the Ethics Committee of the Hospital Clinic of Barcelona (HCB/2021/0242; approved on March 11, 2021). Written informed consent was obtained from all the study participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.