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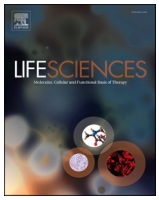

A possible association between low MBL/lectin pathway functionality and microbiota dysbiosis in endometriosis patients

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ABSTRACT

Aims: Endometriosis (EM) is a chronic inflammatory disorder with multifactorial etiologies (*i.e.*, genetics and environmental factors, hormonal and immunological changes, and microbiome alterations). The complement system is one of the most frequently dysregulated pathways in EM. Mannose-binding lectin (MBL), a carbohydrate pattern recognition molecule, is the first described recognition subcomponent of the complement lectin pathway (LP). Here, we unveiled the interplay among MBL polymorphisms, plasma levels, LP functionality, and microbiota as potential contributors to EM pathogenesis.

Materials and methods: A cohort of 38 EM patients and 20 healthy controls was enrolled, and the levels and functionality of the LP were assessed *via* ELISA. MBL genetic variants and the endometrial and vaginal microbiome were investigated and correlated.

Key findings: High MBL levels were related to the disease severity, although not accountable for the *MBL2* genotype. MBL and MASP-2 were present in the uterine mucosa but appeared to have no activity at the endometriotic lesion. EM patients with LP functional deficit displayed pathogenic bacterial species more frequently in the endometrial microbiome. Moreover, women affected by EM showed a higher frequency of rare gene variants in the estrogen pathway genes, potentially affecting MBL plasma levels.

Significance: A lower functionality of LP in the uterine mucosa may contribute to an unbalanced bacterial environment that could activate endometrial cells. Not only the genotype and the inflammatory condition, but also the estrogen pathway can cause altered MBL levels, thus contributing to changes in the LP functionality.

1. Introduction

Endometriosis (EM) is a chronic gynaecological condition in which endometrial tissue grows outside the uterus. The ectopic implantation of functional tissue within the pelvic peritoneum and ovaries leads to recurrent bleeding, nodules, inflammation, pain, scarring, adhesion formation, and eventually infertility [1]. These symptoms can cause anxiety, depression, and impaired social functioning, severely affecting

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the patient's quality of life. It is important to note that EM is a highly prevalent condition, affecting approximately 190 million women globally. It is estimated that nearly 10 % of women are diagnosed with EM in their lifetime [2]. Despite its widespread occurrence, the mechanisms of EM pathogenesis have yet to be fully elucidated. The retrograde menstruation theory, proposed by Sampson in 1927, is most widely recognized but a debatable hypothesis [3,4]; in fact, it fails to account for the existence of extraperitoneal EM.

EM is considered an estrogen-dependent disease; estrogen appears to play a primary role in the development and maintenance of endometriotic lesions, and often, the disease resolves after menopause [5]. EM is considered a multifactorial condition since its onset and progression are influenced by a complex interplay of genetics and environmental factors, hormonal and immunological changes, and microbiome alterations [6,7]. The complement system is one of the most frequently dysregulated pathways in EM [8]. It represents a pivotal component of innate immunity and a bridge to the adaptive immune system, playing a key role in host defense against infectious agents and altered self. The binding of the recognition subcomponents to the target ligands initiates the three different complement pathways: classical (CP; *via* C1q), lectin [LP; *via* Mannan-Binding Lectin (MBL), ficolins, and CL-11 and CL-12 collectins], and alternative (AP; *via* C3) pathways.

MBL, being a humoural C-type lectin belonging to the collectin family, recognizes carbohydrate patterns on the surface of many pathogens, including bacteria, viruses, protozoa, and fungi, thus triggering opsonization and complement activation *via* its association with MBL-associated serine proteases (MASPs) [9,10]. After the first six months of life, MBL levels are highly stable throughout lifetime; the serum levels vary markedly among individuals. Interestingly, >10 % of the Caucasian population can be classified as MBL-deficient. MBL levels can be genetically determined based on three variant allotypes in the exon encoding the collagenous region, termed B, C, and D (codons 52, 54, and 57, respectively), with the wild-type, termed A. The alleles B, C, and D are collectively termed O, and any genotype O/O (*e.g.*, B/B, B/C) results in MBL deficiency. Furthermore, MBL circulating levels are also highly influenced by upstream, non-coding variants [11]. The absence or extremely low concentration of serum MBL seems to be a risk factor for autoimmune diseases [12] and recurrent infections in immunocompromised conditions or before the first 6 months of life [10].

Recent studies on uterine microbiota have challenged the long-held notion that the uterus is an entirely aseptic compartment [13]. The microbiota exerts widespread control over pathogens by directly competing for nutrients and mucosal surface. Furthermore, it plays a crucial role in shaping mucosa, extracting energy, defending against pathogens, and regulating host immunity [14–16]. Alterations in the microbial composition (*i.e.*, dysbiosis) can disrupt these homeostatic functions. Over the past few years, an ever-growing interest has focused on deciphering the composition and functions of uterine microbiota and its potential impact on endometrium activation. Of note, recent advances in genomics and high-throughput sequencing technologies have markedly enhanced our capacity to study the microbiota. One prevalent method involves targeting the bacterial 16S ribosomal RNA (rRNA) gene, which is ubiquitous in all bacteria and archaea. Its nine highly variable regions (V1-V9) facilitate species differentiation [17]. Numerous studies have established a connection between the human microbiota and various inflammatory diseases, including EM [18]. The mechanisms by which the microbiota influences EM may involve several factors (*e.g.*, estrogens, immunity, and inflammation). Recently, a potential infectious contributor to EM has been identified: *Fusobacterium* was found in the endometrium and endometrial lesions of more than half of patients with EM, but only 7 % of controls. Furthermore, it has been demonstrated that TGF-β signaling resulting from *Fusobacterium* infection of endometrial cells led to the transition from quiescent fibroblasts to positive myofibroblasts, which gained the ability to proliferate, adhere, and migrate *in vitro* [19]. Microbiota and innate immunity extensively interact on mucosal surfaces [20]. It has been demonstrated that the activation levels of the complement system in the vagina correlate with the local microbial composition [21]. In addition to the protective effects of the endogenous vaginal/endometrial microbiota, infections by pathogens at this site are prevented by local components of the innate and adaptive immune systems, such as the LP. The exact molecular mechanisms underpinning these interactions are still poorly understood.

In this study, we investigated the interconnection between the LP and microbiota in the pathogenesis of EM. We explored how the functionality of the complement cascade activated by MBL might influence the microbial colonization of endometrium and how uterine dysbiosis could be associated with the development of EM.

2. Material and methods

2.1. Reagents and antibodies

The following antibodies were used: rabbit anti-human MBL (#NBP1–85518) was purchased from Novus Biologicals (Centennial, Colorado, USA); rabbit anti-human MASP-2 (#SC-17905) from Santa Cruz (Dallas, Texas, USA); goat anti-rabbit IgG (H + L) HRP-conjugate (#A120-201P) from Bethyl Laboratories, Inc. (Montgomery, Texas, USA). All other chemicals were purchased from Sigma Aldrich.

2.2. Patient enrollment and specimen collection

Women included in this study were enrolled at the Endometriosis Clinic of the IRCCS "Burlo Garofolo" Hospital (Trieste, Italy). All patients (n = 38) underwent laparoscopic cystectomy of ovarian endometriotic cysts, followed by histological confirmation of the diagnosis. During laparoscopy, endometrial biopsies were obtained using a VABRA aspirator. Right before laparoscopy, a vaginal swab was obtained performing a single gentle 360° rotation of a sterile swab (cliniswab DS 321/SG, APTACA, Canelli, Italy) at the vaginal wall. The severity of EM was assessed using the revised American Society for Reproductive Medicine (rASRM) classification. Upon enrollment, detailed information regarding medical history (*e.g.,* age of menarche, EM diagnosis, family history of EM, number of pregnancies, infertility diagnosis) and EM symptoms (*e.g.*, ovulation, pre-menstrual and post-menstrual pain, dysmenorrhea, dyspareunia, dyschezia, dysuria) were collected.

Tissue samples collected from EM lesions or endometrial biopsies were fixed in 10 % (*v/v*) buffered formalin and embedded in paraffin for immunohistochemistry (IHC) analysis. A section of the lesion was minced and preserved in Trizol (Invitrogen) for total RNA isolation; another section was cut for cell isolation. Before surgery, a whole blood sample was collected in an EDTA-vacutainer tube for ELISA assays.

A control (CTRL) cohort of adult healthy women (n = 20), recruited in the Women4Health project [22], was included in this study. Participants were carefully selected according to the following criteria: 1) a regular menstrual cycle (25–34 days); 2) absence of a clinically and/or surgical diagnosis of EM or polycystic ovary syndrome; and 3) not taking systemic or local hormonal contraceptives in the 30 days prior to the enrollment. A written informed consent for the collection of biological samples for research purposes was available for all participants. In particular, for this study, a whole blood sample was collected in an EDTA-vacutainer tube for ELISA and a salivary sample was taken for DNA extraction.

The study was approved by the Ethics Committee of the Friuli-Venezia Giulia region (Italy; Protocol n. 0023422/P/GEN/ARCS 2021), and all participants provided written informed consent to participate.

2.3. Evaluation of complement pathways functionality

The activation of CP, AP, and LP was measured using the commercial Wieslab® kit (Technogenetics, Milan, Italy). With this ELISA kit, the

term "LP" refers to the evaluation of the functionality of MBL, MASP-1, and MASP-2, but not of ficolins. The assay was performed following the manufacturer's instructions. The absorbance was read at 405 nm by the PowerWave X Microplate Reader (Bio-Tek Instruments) spectrophotometer.

2.4. ELISA

The human MBL ELISA kit (R&D, Minneapolis, USA) and the human MASP-2 ELISA kit (Hycult, Milan, Italy) were used to quantify MBL or MASP-2, respectively, in plasma samples, following manufacturer's instructions. The microtiter plate was read at 450 nm by the PowerWave X Microplate Reader (Bio-Tek Instruments) spectrophotometer.

2.5. Whole-exome sequencing analyses

For each EM patient, whole-exome sequencing (WES) data were already available *via* an *in-house* database [23]. For each participant in the CTRL cohort, genomic DNA was extracted from a salivary sample using the DNA Genotek kit (OrageneTMDNA collection kit, DNAGenotek, Inc., Ottawa, Canada). The DNA quality was evaluated through 0.8 % (*w/v*) agarose gel electrophoresis, and the concentration was estimated at OD_{260} using the Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). WES analysis was performed employing the Illumina NextSeq 550 System (Illumina Inc., San Diego, CA, USA) with the Twist Exome 2.0 plus Comprehensive Exome Spike-in kit (Twist Bioscience, San Francisco, CA, USA), following the manufacturer's protocol. WES secondary and tertiary analyses were carried out, as previously described by Santin *et al.* [23].

2.6. Complement system and estrogen pathway gene variants extraction

Two gene lists were created for WES data analysis (**Supplementary Tables 1** and **2**). The first one was generated following the literature review and comprised nine genes (*i.e.*, *FCN1, FCN2, FCN3, MBL2, MASP1, MASP2, C2, C3,* and *C4*), known to be involved in the complement pathways **(Supplementary Table 1**).

The second list included 44 specifically selected genes, all involved in the estrogen signaling pathway and known to modulate 17-β estradiol levels. Specifically, this list was created according to the following steps: i) the "estrogens signalling pathway" genes lists provided by Ingenuity Pathway Analysis software (IPA, Ingenuity System Inc., USA; https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) and KEGG PATHWAY Database (https://www.genome.jp/kegg/pathway.html) were combined together; ii) employing IPA "Path explorer" function, only the genes known to modulate 17-β estradiol levels were retained. Genes belonging to the Cytochrome P450 and UGT gene family have been excluded. The complete list is provided in **Supplementary Table 2**.

Variants with a MAF cut-off of 0.1 % and predicted damaging by *in silico* prediction tools, such as PolyPhen-2 [24], SIFT [25], PaPI [26], DANN [27], dbscSNV score [28] and SpliceAI [29], were considered. Variants with a Variant Allele Frequency < 30, quality score < 20, SNVs leading to synonymous amino acid substitutions, not affecting splicing, highly conserved residues or located in off-target regions were discarded from the analyses. Sanger sequencing was performed to confirm all the selected variants. DNA was analyzed on a 3500 Dx Genetic Analyzer (ThermoFisher, Waltham, MA, USA), employing the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Waltham, MA, USA).

2.7. MBL2 genotypes extraction

Employing *bcftools* (version 1.4) software [30], the following *MBL2*-related single nucleotide polymorphisms (SNPs) were extracted from VCF files: rs5030737 (allele D), rs1800450 (allele B), and rs1800451 (allele C). The alternative alleles of these SNPs are collectively named O, while the wild-type allele as A [31]. For each EM patient and healthy CTRL, the genotypes relative to rs1800450, rs1800451, and rs5030737 were determined. The frequency of the extracted genotypes (*i.e.*, A/A, A/O, O/O) was then compared between the two cohorts employing a sample test of proportions, and a binomial test. The statistical significance was set to p-value (p) < 0.05, and the analysis was conducted with R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

2.8. High-resolution melting analysis

The primers used for *MBL2* amplification were previously described by Arraes *et al.* [32]. PCR reactions for High-Resolution Melting Analysis (HRMA) were performed with Type-it HRM PCR Kit (Qiagen) using the Corbett Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). The reaction mix contained 20 ng of genomic DNA, 0.7 μM of forward primer (5'-AGGCATCAACGGCTT CCCA-3′), and 0.6 μM of reverse primer (5'-CAGAA CAGCCCAACACGTACCT-3′). Cycle conditions were: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. After amplification, the samples were cooled for 1 min at 50 °C to allow heteroduplex formation, then melting curve data were acquired by slowly increasing the temperature from 75 °C to 90 °C at a rate of 0.1 °C/5 s. Data were acquired and analyzed using software provided with the RotorGene TM 6000 Instrument. Each sample was tested in duplicate.

2.9. Gene expression profiling (GEP) analysis

The analysis of *MBL2* and *MASP2* gene expression in control endometrium and different EM lesions [*i.e.,* peritoneal, deep, and ovarian endometrioma (OMA)] was conducted using data obtained from the Gene Expression Omnibus of the National Centre for Biotechnology Information (NCBI) with the accession number GSE141549. Microarray analysis on samples obtained from 43 endometrium biopsies of healthy women, 101 endometrium biopsies of EM patients, and 190 EM lesions, and data normalization as well, have been described by Gabriel and colleagues [33]. Stages of EM were defined following the rASRM classification [34].

2.10. Gene expression analysis

Tissue samples from endometriotic lesions were minced and lysed using Trizol reagents (1 mL for 50–100 mg of tissue). Total RNA extraction was carried out using the PureLink™ RNA Mini Kit (Invitrogen, ThermoFisher). For isolated primary cells, RNA extraction was performed by lysing the cells with RNA Lysis Buffer and using the Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada), following the manufacturer's protocol. Isolated RNA was then quantified using the NanoDrop™ 2000/2000c spectrophotometer (ThermoFischer Scientific, Massachusetts, USA) and reverse transcribed into cDNA using the SensiFAST™ cDNA Synthesis kit (Meridian Life Science, Memphis, TN, USA). For RT-qPCR, the Power SYBR™ Green Master Mix (Applied Biosystems, Life Technology, USA) was used, and the reaction was performed using the Corbett Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). Expression levels of the human *MBL2*, *MASP2*, and *C3* genes were assessed through a comparative quantification analysis, considering reaction efficiency and normalization against the housekeeping gene, *GAPDH* (Table 1).

2.11. Immunohistochemistry

Tissue samples were fixed in 10 % *v/v* buffered formalin and paraffin-embedded. 4-5 μm tissue sections were deparaffinized with xylene and rehydrated with decreasing ethanol dilutions (100 %, 95 %, 70 %) and H_2O. Antigen retrieval was performed for 20 min at 98.5 °C in Tris-HCl/EDTA buffer, pH 9.0, for MASP-2, and in citrate buffer, pH 6.0 for MBL staining. Neutralization of the endogenous peroxidases was

Table 1
List of primers used for Real-Time quantitative PCR.

Gene	Melting Temperature (C°)	Forward sequence Reverse sequence	Accession number
MBL2	60	5'-GCCTCAGAAAGAAAAGCTCTGC-3′ 5'-TGGTCAGGAAGAACTTGTTCCC-3′	NM_000242.3
MASP2	60	5'-TGCAGCATTGTTGACTGTGG-3′ 5'-AGCTTTGTAGGTGGTCACTCC-3'	NM_006610.4
C3	60	5'-CCTGCTACTAACCCACCTCC-3′ 5'-AACAGTGACTGGAACATCCCC-3'	NM_000064.4
GAPDH	60	5'-TCTCTGCTCCTCCTGTTC-3′ 5'-GCCCAATACGACCAAATCC-3'	NM_001357943.2

performed by adding H_2O_2 for 5 min, and blocking of nonspecific binding was carried out *via* incubation with PBS + 2 % *w/v* BSA for 30 min. Sections were incubated with primary antibodies, rabbit anti-human MASP-2 (1:75) or rabbit anti-human MBL (1:500), overnight at 4 °C, followed by incubation with anti-rabbit HRP-conjugated (1:500) for 30 min at RT. Staining was performed by 3-amino-9-ethylcarbazole (AEC) chromogenic substrate (Vector Laboratories). Sections were counterstained with Mayer hematoxylin (DiaPath), and examined under a Leica DM 2000 optical microscope. Images were acquired using a Leica DFC 7000 T digital camera (Leica Microsystems, Wetzlar, Germany).

2.12. 16S rRNA gene sequencing

Total DNA was extracted from 100 mg of each biopsy in a final elution volume of 50 μL and from 300 μL of vaginal swab in a final elution volume of 50 μL by the automatic extractor Maxwell CSC DNA Blood Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

The microbial profiling was performed by sequencing the region V3 of the 16S rRNA gene. First, a V1-V3 region of the 16S rRNA gene (500 bp) qPCR was carried out, with the primer set, U534R and 27FYM. Afterwards, a semi-nested V3 region (200 bp) qPCR was performed with the primer set B338F_P1-adaptor and U534R_A_adaptor_barcode, using a different barcode for each sample. The PCR reactions were set as follows: EvaGreen® dye (Fisher Molecular Biology, Waltham, MA, USA), Kapa 2G HiFi Hotstart ready mix 2× (Kapa Biosystems, Wilmington, MA, USA), 0.5 μM of each primer, and 400 ng/μL of BSA, in a final volume of 10 μL. The PCR cycle parameters were: 5 min at 95 °C, 30 s at 95 °C, 30 s at 59/57 °C, 45 s at 72 °C, and a final elongation/extension step at 72 °C for 10 min. No template controls, in parallel with clinical samples starting from the pre-analytic phase, were considered as negative controls. The size of the amplicons (560 bp for the first PCR and 260 bp for the semi-nested PCR) was checked on a 2 % agarose gel. The Qubit® 2.0 Fluorimeter (Invitrogen, Carlsbad, California, USA) was used to quantify the amount of dsDNA in each sample after the semi-nested PCR using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). An equal amount of each sample (100 ng) was mixed into a single batch to generate a pooled library at a final concentration of 100 pM, according to the manufacturer's instructions. Template preparation was performed by emulsion PCR using the Ion OneTouch™ 2 System (Life Technologies, Gran Island, NY, USA), with the Ion PGM Hi-Q View OT2 kit (Life Technologies, NY, USA), and subsequent quality control was carried out using a Qubit® 2.0 Fluorimeter. Sequencing was performed with the Ion PGM™ System technology *via* Ion PGM Hi-Q View sequencing kit (Life Technologies, NY, USA).

2.13. 16S rRNA gene sequencing data analysis

The FastQ files derived from the V3—16S rRNA gene sequencing were processed using QIIME 2.0 (version 2022.2), filtering out reads with $Q < 20$ and a read length < 180 bp, after DADA2 denoising. Silva v138 was chosen for the taxonomy assignment, with a BLAST+ consensus. Before further analysis, the reads were rarefied to even read count, after filtering out those reads overlapping the no template controls and those unassigned or assigned as human. The Shannon (value = 1 when all species have the same abundance) and observed ASVs (the total number of species in the samples) metrics were calculated to assess the alpha diversity (microbiome diversity within a community), and compared by means of the Kruskal–Wallis test. The Bray–Curtis dissimilarity index was calculated to assess the beta diversity, which measures the similarity or dissimilarity of the analyzed groups, visualized by the principal coordinate analysis (PCoA), and compared by the PERMANOVA test. To highlight the differences in the microbial composition, we performed differential abundance testing using the ANCOM test. Using MicrobiomeAnalyst, we applied the LEfSE test to identify microbial biomarkers [35].

2.14. HepG2 stimulation

HepG2, a hepatocarcinoma cell line, was kindly provided by Dr. Cristina Bellarosa (Italian Liver Foundation, Trieste). The cells were cultured in high-glucose DMEM (Gibco Life Technologies, Milan, Italy) containing 10 % *v/v* heat-inactivated FBS (Gibco Life Technologies), 1 % *v/v* penicillin/streptomycin, (Gibco Life Technologies) in a humidified atmosphere of 5 % CO_2 at 37 °C. The culture medium was replenished every 2–3 days, and cells were passaged every 7 days. 2.5×10^5 cells were seeded in 12 well plate and stimulated for 24 h with different concentrations of β-Estradiol-Water Soluble (E4389, Sigma-Merck). RNA extraction was performed by lysing the cells with RNA Lysis Buffer and using the Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada), following the manufacturer's protocol. Isolated RNA was then quantified using NanoDrop™ 2000/2000c spectrophotometer (ThermoFischer Scientific, Massachusetts, USA) and reverse transcribed into cDNA using SensiFAST™ cDNA Synthesis kit (Meridian Life Science, Memphis, TN, USA). For RT-qPCR, Power SYBR™ Green Master Mix (Applied Biosystems, Life Technology, USA) was used and the reaction was performed using Corbett Rotor-Gene™ 6000 (Qiagen, Hilden, Germany). Expression levels of the human *MBL2* and *MASP2* genes were assessed through a comparative quantification analysis, considering reaction efficiency and normalization against the housekeeping gene, *GAPDH*.

2.15. Statistics

Data were analyzed by GraphPad Prism software 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Complement system functionality and analysis of gene expression (HepG2 stimulation) were analyzed using unpaired non-parametric Mann-Whitney test. For GEP analysis and ELISA quantification (normal distribution was determined with Shapiro-Wilk test), unpaired *t*-test was applied. Results were expressed as box and whiskers. The experiments were performed at least in duplicate, and $p < 0.05$ were considered statistically significant. In this pilot monocentric study, a formal sample size calculation has not been performed.

3. Results

3.1. Clinical features of EM patients

A group of 38 EM patients, with a mean age of 37 (± 8.29) years, and a menarche age of 12.1 (± 1.45), were enrolled at the IRCCS "Burlo

Garofolo" Hospital (Trieste, Italy). The demographic data and clinical features of the subjects who participated in the study are summarized in Table 2. The ethnicity of all control healthy women (CTRL) was Caucasian.

According to the rASRM standard classification [34], 86 % of the patients were diagnosed with stage III-IV. Further, at enrollment, about 16 % of the EM patients received a diagnosis of infertility, while 59 % of patients were taking hormonal therapies (*i.e.,* progestins, combined estrogen–progestins, or hormone-releasing intrauterine devices).

3.2. Evaluation of complement system functionality and MBL/MASP levels in EM vs CTRL women

To investigate the LP activation, particularly its activators MBL/MASP in EM, the functionality of CP, AP, and LP were assessed in the EM cohort of patients ($n = 38$) enrolled at the IRCCS "Burlo Garofolo" Hospital (Trieste, Italy). As shown in Fig. 1A, both CP and AP presented standard functionality in EM women, whereas a high percentage of functional deficiency was found for LP (39.5 %) (Fig. 1B). However, these results did not mirror a statistically significant difference compared to a group of matched healthy women (CTRL, 30 %; $n = 20$).

In the same samples, the analysis of MBL levels showed a significant variation between EM and CTRL groups (F test; $p < 0.01$) (Fig. 1C). This difference can be explained by the observation that one group of women displayed extremely high MBL levels among EM patients (13–15 %); for instance, six patients had MBL values >2 up to 2.84 μg/mL. The remaining patients had very low or absent MBL (25 %): ten patients had levels below the 25th percentile (0.45 μg/mL); among these, three patients were defective (8 %) (Fig. 1D). This large number may partly explain the high percentage of LP-deficient cases in the EM group. Conversely, no difference was observed in MASP-2 circulating levels between EM and CTRL groups (Fig. 1E). Interestingly, the clustering of EM patients based on disease severity (*i.e.*, I-III stages and IV stage) revealed a significant difference in the MBL expression between the two groups (I-III stages *vs* IV stage), with a higher level of MBL in the most severe group (Fig. 1F); however, in this case, no difference was observed for MASP-2 levels (Fig. 1G).

Table 2
Demographic characteristics of the enrolled EM patients.

EM Patients ($n = 38$)	mean ± SD	
Age, years	37.0 ± 8.29	
Age at menarche, years	12.1 ± 1.45	
Body-mass index, kg/m^2	22.84 ± 3.56	
Ethnicity	***n***	**%**
Caucasian	35	92 %
Hispanic	2	5.3 %
African	1	2.6 %
Obstetrics and gynaecological data	***n***	**%**
Patients with infertility diagnosis	6	15.7 %
Patients reporting spontaneous miscarriages	8	21.1 %
Surgery	***n***	**%**
Yes	38	100 %
No	0	0 %
rASRM classification	***n***	**%**
Stage I	1	3 %
Stage II	4	11 %
Stage III	12	31 %
Stage IV	21	55 %
Ongoing medical therapy	***n***	**%**
Yes	23	60 %
No	15	40 %
CTRL ($n = 20$)	**mean ± SD**	
Age, years	27.40 ± 5.71	
Body-mass index, kg/m^2	21.95 ± 2.75	

Stages were defined following the revised American Society for Reproductive Medicine (rASRM) classification [34]. Abbreviations: *n*, number; SD, standard deviation.

3.3. Complement gene variant extraction from WES analysis and MBL polymorphisms in patients with EM

To further evaluate the functionality of the complement system in the EM cohort, Whole-Exome Sequencing (WES) analysis was carried out, focusing on nine complement system-associated genes involved in the activation of LP (**Supplementary Table 1**). Only in 2/38 (5 %) patients, two different rare predicted damaging variants within the *MASP2* gene were identified. In particular, patient #42, displaying low LP activity and high MBL levels (based on ELISA results), carried a novel frameshift variant, while in patient #64, displaying no LP activity and normal MBL level, a missense variant was detected (**Supplementary Table 2**). Both variants resulted in a low or no LP functionality.

Several polymorphisms have been previously identified in the *MBL2* gene. However, three SNPs are particularly notable, being in close proximity to each other: at codon 52 (rs5030737, C > T, Arg > Cys), 54 (rs1800450, G > A, Gly > Asp), and 57 (rs1800451, G > A, Gly > Glu) [10,11]. For each EM patient and CTRL, the previously reported *MBL2*-related SNPs were extracted from WES data (**Supplementary Tables 3** and **4**). In particular, 4/38 (11 %) EM patients, and 4/20 (20 %) CTRL individuals carried the SNP rs5030737. Regarding rs1800450, the heterozygous carriers were 13/38 (35 %) for the EM cohort, and 5/20 (25 %) for the CTRL group, while only one patient of the CTRL group carried both alternative alleles at this position. Finally, 1/38 (2.6 %) patients of the EM cohort and 1/20 CTRL individuals (5 %) were identified as heterozygous carriers of rs1800451. However, due to the low coverage quality, it was impossible to define the genotype at rs1800451 for 4/38 (11 %) of EM patients.

Overall, according to WES data analysis, 20/38 (54 %) patients of the EM cohort and 12/20 (60 %) of the CTRL cohort showed the A/A genotype, 16/38 (43 %) EM patients and 4/20 (20 %) CTRLs presented the A/O genotype, while one EM patient (2.7 %) and four CTRLs (10 %) carried the O/O genotype. When the frequency of A/A, A/O, and O/O genotypes between the EM and CTRL cohort was evaluated, no statistical differences were observed between EM and CTRL women ($p > 0.05$).

Finally, we utilized HRMA, a simple and powerful technique for SNP detection, to analyze *MBL2* gene (**Fig. 2A** and **B**) and confirm the three potential genotypes A/A, A/O, and O/O in both EM and CTRL groups. As shown in **Fig. 2C** and **D**, our results were in accordance with MBL plasma levels (except for a few patients) but not completely with LP activity (**Fig. 2E** and **F**). However, HRMA confirmed the presence of 2.7 % O/O and 43.2 % A/O in our cohort of EM patients (**Fig. 2G** and **H**).

3.4. Distribution of MBL and MASP-2 in endometriotic lesions and eutopic endometrium

To further dissect the role of the MBL/MASP-driven LP in EM, we interrogated the publicly available database EndometDB, developed by Turku University (Finland), which collected gene expression data from 190 EM patients and 43 healthy controls [33]. GEP analysis showed very low expression of *MBL2* and *MASP2* transcripts in all EM histotypes (peritoneal, deep, and OMA), such as in healthy control endometrium (CE) and patient endometrium (PE) (**Fig. 3A** and **B**), revealing no differences between eutopic and ectopic tissues.

Subsequently, we isolated total mRNA from endometriotic ovary lesions. In accordance with EndometDB, gene expression analysis using Real-Time quantitative PCR (RT-qPCR) revealed no local expression of *MBL2* or *MASP2* in all analyzed EM tissues (Fig. 3D). The expression was compared to *C3* based on previous evidence [36]. The hepatocarcinoma cell line, HepG2, was used as a positive control and calibrator. We investigated the local distribution of MBL and MASP-2 in normal and ectopic endometrium by performing IHC in tissue samples. As shown in Fig. 3E, the eutopic endometrium of EM patients was weakly positive (particularly in the gland lumen), whereas EM lesions were almost negative for MBL staining (Fig. 3F). Surprisingly, in both healthy and

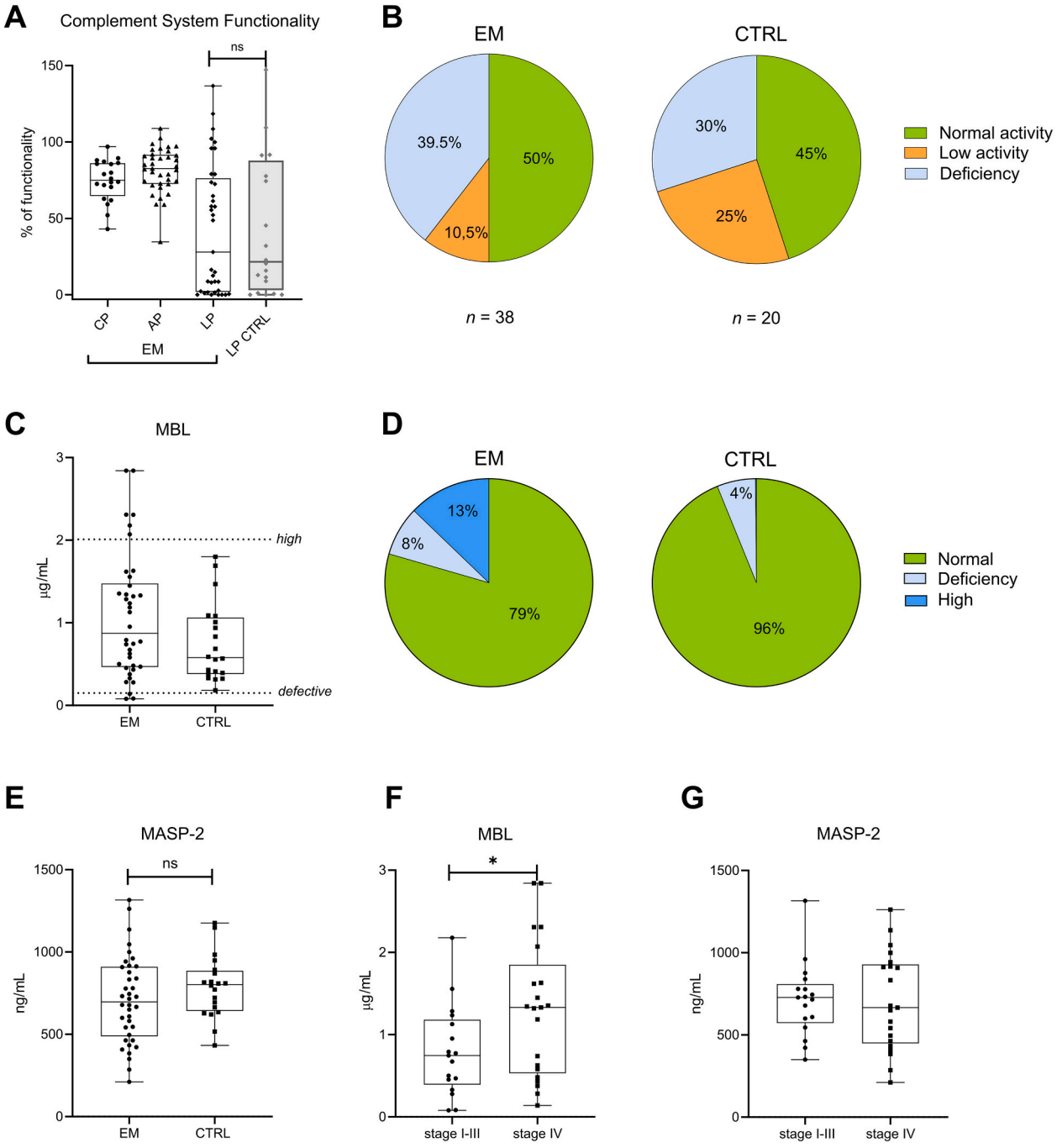

Fig. 1. Characterization of the LP in EM patients. (**A**) Evaluation of the functionality percentage of classical (CP), alternative (AP), and lectin (LP) pathways in EM patients *via* Wieslab® ELISA kit. The functionality of LP of EM patients was compared with that of a healthy control group of women (LP CTRL). (**B**) Pie graphs representing the percentages of normal, low, or deficient functionality of LP in EM ($n = 38$) and CTRL ($n = 20$) groups. (**C**) Measurement of circulating levels of MBL in the plasma of EM patients and CTRL group. (**D**) Pie graphs representing the percentages of women with high, normal, or low plasma concentrations of MBL in EM and CTRL groups. (**E**) Measurement of circulating levels of MASP-2 in the plasma of EM patients and CTRL group. (**F-G**) Analysis of MBL (**F**) or MASP-2 (**G**) circulating levels in EM patients clustered into different disease stages (stage I-III or IV). MBL levels were significantly higher in stage IV patients. $^*p < 0.05$; *ns,* not significant (*t*-test).

pathologic tissues, we detected strong staining for MASP-2, especially in the cells of endometrial origin (Fig. 3**H** and **I**). The staining of the human liver, used as tissue control, showed an intense positivity for both complement components (Fig. 3**G** and **J**).

3.5. Influence of LP functionality on microbiome dysbiosis of the reproductive tract

Of 38 enrolled patients, 22 endometrial biopsies were available for microbial profiling. Five of these biopsies (*i.e.*, patients #58, #59, #76, #78, and #81) showed no presence of bacterial DNA (Fig. 4A). The uterine samples that tested negative for bacterial DNA contained 'unassigned' reads, primarily from mitochondrial or human sources, or

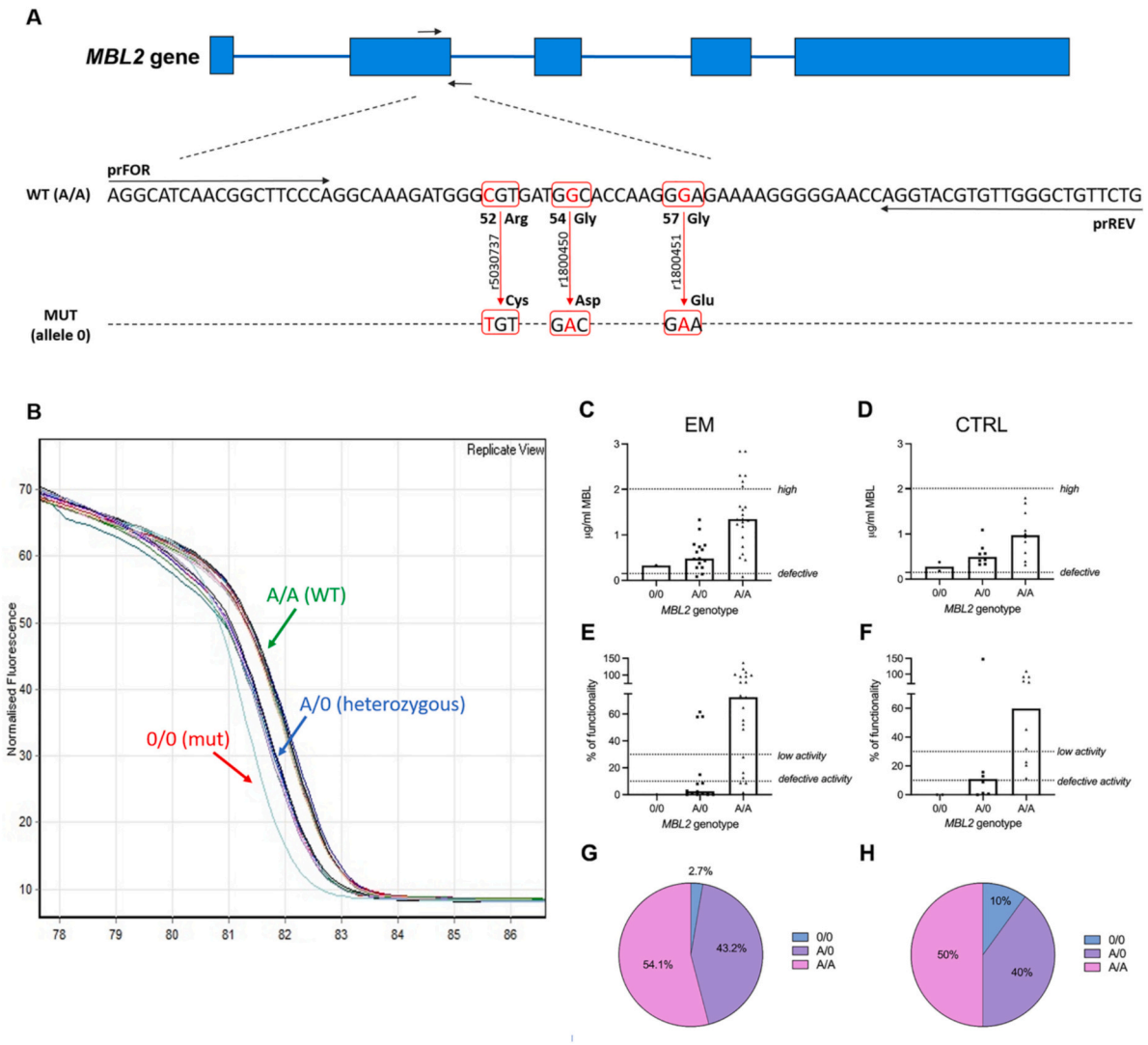

Fig. 2. Characterization of *MBL2*-associated polymorphisms in EM patients. (**A**) Diagram of human *MBL2* gene structure and the primer positions and directions (black arrows). Blue boxes represent exons. Below amplified sequence, position of the three mutations, and SNPs are highlighted in red. (**B**) Representative normalized plot for the genotype of A/A (wild-type, WT), A/O (heterozygous), and O/O (mutated, mut). It is possible to distinguish the three genotypes by their different melting curve shape. (**C, D**) Analysis of circulating levels of MBL in EM (**C**) and CTRL (**D**) groups clustered into MBL genotypes. (**E, F**) Analysis of the functionality of the lectin pathway in EM (**E**) and CTRL (**F**) groups clustered into MBL genotypes. (**G, H**) Pie graphs representing the percentages of A/A, A/O, and O/O MBL genotypes in EM (**G**) and control (**H**) groups.

overlapping reads with the no-template controls, which indicate contaminants from sequencing reagents. After the quality filtering step, the total read count was 523,857 with 23,811 (6904–115,873) average counts per sample. According to alpha/beta diversities and ANCOM/LEfSE tests, there were no significant differences based on the LP functionality and MBL circulating levels at the phylum, genus, and species levels (**Supplementary Figs. 1** and **2**). However, a trend could be observed in this cohort at the genus level. A defective LP functionality was observed alongside a more heterogeneous and dysbiotic microbiome compared to the other two groups (low and normal LP functionality) (Fig. 4B). In particular, bacteria such as *Gardnerella, Prevotella, Atopobium, Streptococcus,* and *Escherichia-Shigella* were present. When Lactobacilli were spotted as the predominant or unique bacterial genus in the endometrium, the functionality of LP resulted as low or normal.

Regarding the vaginal swabs, after the quality filtering step, the total reads count was 1,710,552 with 77,752 (24867–210,559) average counts per sample. The profiling of the vaginal microbiome highlighted an opposite situation compared to the endometrium (*i.e.,* a higher dysbiosis in patients with normal functionality of the LP) (Fig. 5A), leading us to conclude that LP is not essential for maintaining the balance of the microbial microenvironment in the vaginal mucosa. Indeed, its activation could increase the local inflammatory state by interfering with the most microbially correct colonization. Interestingly, the comparison of vaginal and endometrial microbiomes in individual patients demonstrated a tendency for the LP-sufficient individuals who present vaginal dysbiosis to "correct" the microbiome composition in the uterine mucosa. This observation seems in accordance with the lowest number of shared bacteria between the vagina and the endometrium in LP-sufficient individuals compared to the women with defective and low LP activation (Fig. 5B).

3.6. *17-βestradiol levels influence the synthesis of MBL by hepatocytes*

As the difference in MBL concentrations between EM and CTRL groups could not be attributed solely to the MBL genotype, we assessed the presence of potential modulatory factors affecting its expression. Since EM is not only considered a chronic inflammatory but also an estrogen-dependent disease, and since hepatocytes are the primary

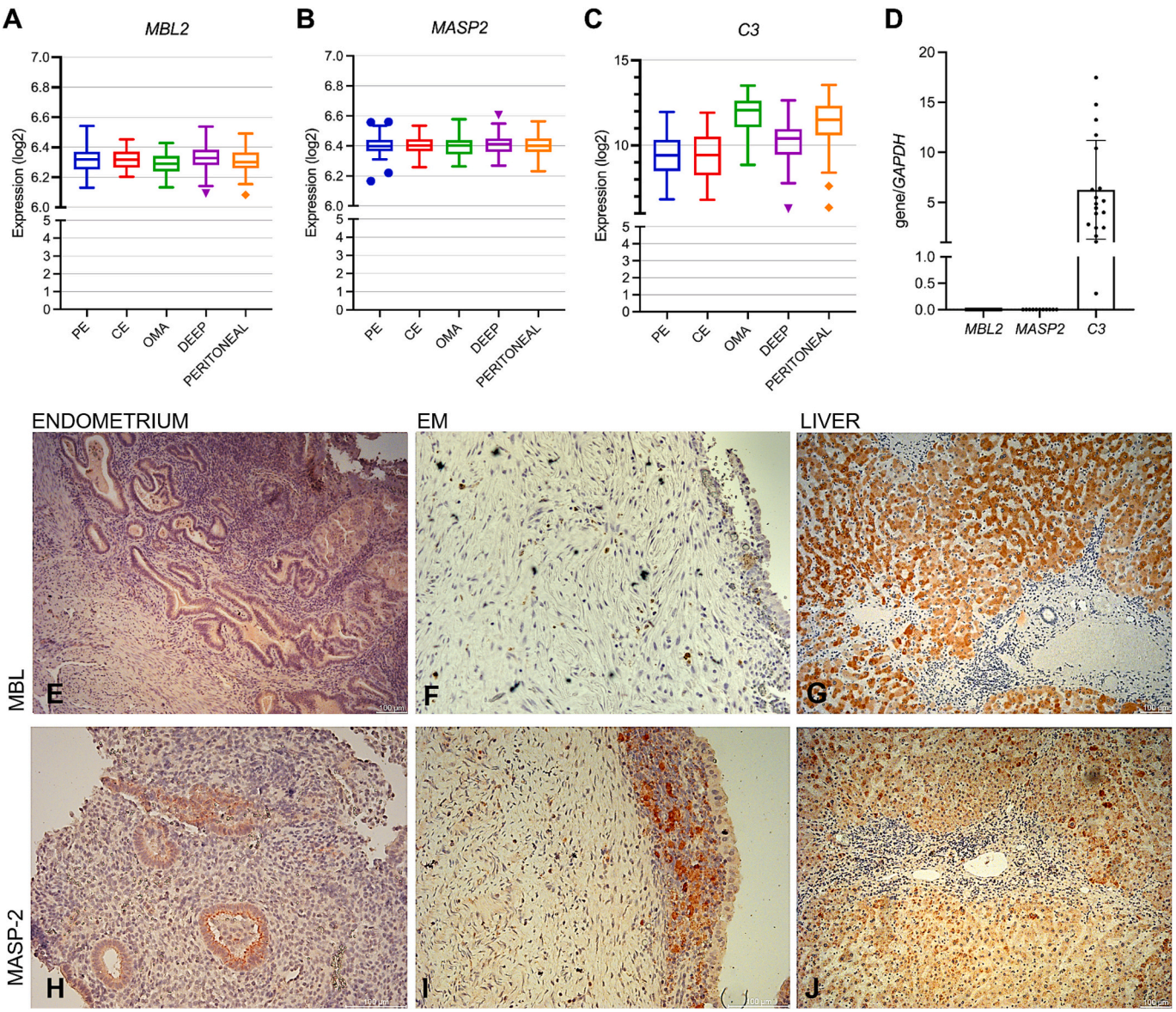

Fig. 3. Characterization of MBL and MASP-2 expression at tissue level. (**A-C**) Histograms representing *MBL2* (**A**), *MASP2* (**B**), and *C3* (**C**, as positive control) mRNA expression in control endometrium (CE), patient endometrium (PE), and in different EM lesions (peritoneal; deep; and ovarian, OMA). Gene expression profiling (GEP) analysis, based on the data extracted from GEO (GSE141549), revealed a very low expression of *MBL* and *MASP2* transcripts compared to *C3* mRNA. (**D**) After total RNA extraction and reverse-transcription, *MBL2* and *MASP2* gene expression was analyzed by RT-qPCR. *C3* gene expression analysis was conducted as the positive control. The hepatocarcinoma cell line HepG2 was used as positive control and calibrator. *GAPDH* was used as the housekeeping gene. No expression of *MBL2* and *MASP2* was observed in EM tissues. (***E*-J**) Representative microphotographs showing the presence of MBL or MASP-2 in patients' eutopic endometrium (**E, H**), ovarian endometriotic lesions (EM) (**F, I**) or liver (**G, J,** as control tissue). AEC (red) chromogen was used for primary antibody visualization. Nuclei were counterstained in blue with Harris Hematoxylin. Magnification, 10× (**E, G, J**); 20× (**F, H, I**). Scale bars, 100 μm.

source of circulating complement components, we investigated the role of 17-β estradiol (E2) in the modulation of MBL (or C3 as control) gene expression by HepG2, a hepatocyte cell line. HepG2 cells were grown to confluence, stimulated with different concentrations of 17β-estradiol for 24 h, and analyzed for the expression of *MBL2* by RT-qPCR. Our results indicated that at 100 pg/mL, the hormone induced a downregulation of *MBL2* expression. Conversely, at the concentration of 350 pg/mL, it induced an upregulation of the *MBL2* gene transcription (**Supplementary Fig. 3 A**). No E2 concentrations were able to modify the expression of *C3* significantly (**Supplementary Fig. 3B**). This observation, together with the inflammation hypothesis, can explain the elevated levels of MBL in stage IV EM patients, but not the elevated percentage of MBL-deficient EM patients compared to healthy control women.

3.7. Identification of rare variants within estrogens signaling pathway genes

To further analyze the role of E2 in regulating MBL expression, WES data analysis was focused on a selected list of 44 estrogen pathway-associated genes, specifically involving those genes described as modulators of 17-β estradiol levels (**Supplementary Table 2**).

Overall, WES data analysis allowed the detection of seven rare (minor allele frequency, MAF < 0.1 %), predicted damaging variants within seven genes, namely *TGFB3*, *NCOA4, RBMS1, SULT1E1*, *BRD2*, *GCG*, and *NR1I2,* in 6/38 (16 %) of the analyzed EM patients (**Supplementary Table 5**). All variants were detected in the heterozygous state.

Specifically, patient 35 carried a missense variant within *TGFB3* gene, which has been described to increase E2 release from primary

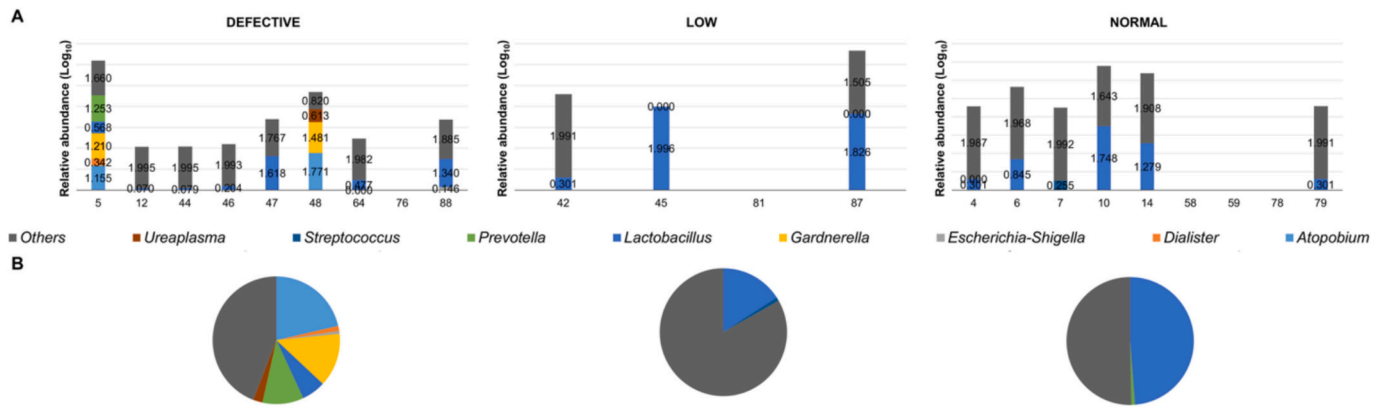

Fig. 4. Microbial profiling of the endometrium in EM patients. (A) Bar plots represent the $\log_{10}$-transformed relative abundance of the bacterial genera identified in each analyzed endometrial biopsy ($n = 22$). Some samples ($n = 5$) showed no bacterial DNA (missing columns in the bar plots). (**B**) Pie charts represent the mean relative abundances of the bacterial genera of the samples grouped based on the defective, low, and normal LP functionality.

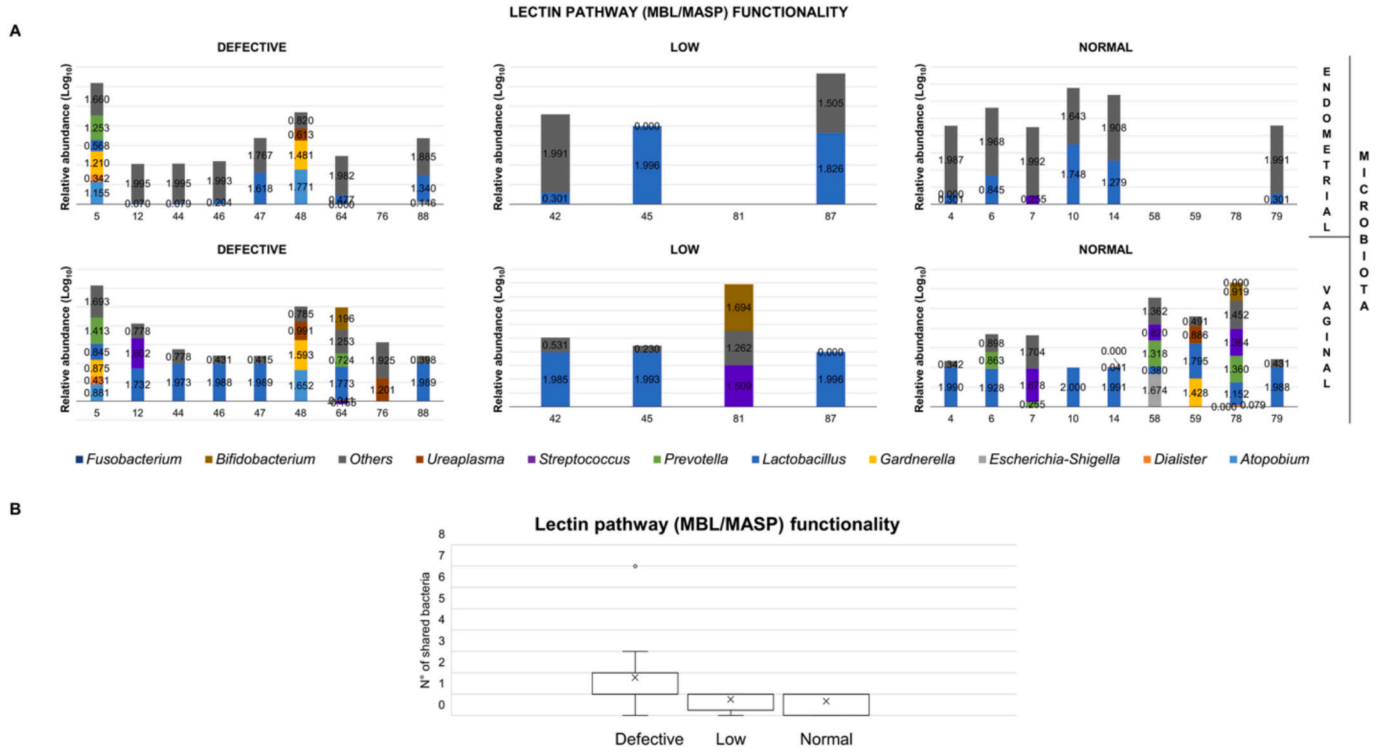

Fig. 5. Comparison between vaginal and endometrial microbiome in EM patients. (**A**) Bar plots show the log10-transformed relative abundance of the bacterial genera identified in each analyzed vaginal swap (lower panel) and endometrial biopsy (upper panel). Some samples showed no bacterial DNA (missing columns in the bar plots). (**B**) Number of shared bacterial genera between the vagina and the endometrium in EM patients grouped according to the lectin pathway functionality.

granulosa cells of ovarian follicles in murine models [37]. Furthermore, patient 38 carried a predicted damaging deletion within *NCOA4* gene that encodes the Nuclear receptor co-activator 4 protein. In patient 46, a nonsense variant was detected within *RBMS1* gene, previously characterized for its involvement in E2 release from ovarian granulosa cells in mice models [38]. Moreover, patient 47 was carrier of a truncated variant within *SULT1E1* gene, encoding the estrogen sulfotransferase, an enzyme that sulfates estrogens and regulates their homeostasis [39]. Regarding patient 48, a missense variant was discovered within *BRD2* gene, encoding a transcriptional regulator of estrogens-modulated genes [40]. Lastly, two different genetic variants within two genes have been identified in patient 64: 1) *GCG* gene, whose protein product (*i.e.*, glucagon-like peptide-1) was found to reduce E2 secretion in mice models [41], and 2) *NR1I2* gene, encoding the Pregnane X receptor, known to be involved in estrogen metabolism regulation [42].

Five of these six patients were characterized by MBL/LP deficiency (**Supplementary Table 5**). This observation led to the hypothesis that these rare pathological variants within estrogen signaling-related genes could impact proper gene function, thus influencing E2 levels and subsequent MBL secretion by hepatic cells.

4. Discussion

Immunological dysfunction, particularly inadequate complement activation, may play a prominent role in the pathogenesis of EM [8]. MBL and MASP-2 participate in complement LP activation when MBL binds carbohydrate patterns found on the surface of micro-organisms [43]. MBL can activate the immune system through LP; however, it

can also perform innate immune functions directly as an opsonin without involving complement pathway, such as enhancement of phagocytosis, stimulation of the secretion of pro-inflammatory cytokines such as IFN-γ, IL-17, IL-6, and TNF, as well as the production of NO by macrophages. This helps limit infection while orchestrating subsequent adaptive immune response [44].

Our results showed no statistical differences between EM and CTRL women in MBL/LP functionality, even though we observed a noticeable difference in the percentage of women with deficient and low MBL levels in the EM and control groups. The analysis of MBL levels revealed a significant difference in variation and data distribution (*F* test) between EM and CTRL groups. This result can be attributed to the evidence that the EM group was divided into two subgroups: one with high MBL levels and the other with very low or absent levels. Furthermore, our results indicate a correlation between MBL levels and EM severity. This observation, apparently at odds with the LP functionality data, is in accordance with the evidence that EM is a condition characterized by chronic inflammation, and MBL is a serum acute-phase protein secreted by the liver [45]. Thus, high MBL is not the primary cause of EM onset but the inflammatory condition of severe EM that determines MBL levels' ele. Recently, Jensenius' group, in a case-control study of blood samples from 100 patients with EM compared to 350 blood donors, demonstrated no association between EM and low levels of MBL. Partially in contrast to our results, Sikora et al. showed that MBL did not differ in concentration in the peritoneal fluid of patients with and without EM, despite its role in modulating inflammation and enhancing the clearance of apoptotic cells [46].

Genetic MBL insufficiency has an important impact on the host defense, particularly during the vulnerable period of childhood from 6 to 17 months, when the adaptive immune system is not completely developed [47]. To fully understand the role of MBL and LP in EM pathogenesis, WES data analysis was performed to extract nine complement system-associated genes. In 5 % of patients, two different rare predicted damaging variants within the *MASP2* gene were identified, both resulting in low or absent LP functionality. The remaining patients were negative for this analysis. According to WES data analysis, 54 % of patients in the EM cohort and 58 % of the healthy controls showed the A/A *MBL2* genotype; 43 % of the EM patients and 33 % of controls presented the A/O genotype, while one EM patient and four healthy controls carried the O/O genotype. To compare the frequency of A/A, A/O, and O/O genotypes between the EM and CTRL cohort, a sample test of proportions for the first two genotypes and a binomial test for the O/O genotype were performed. In accordance with Kruse and colleagues [48], no statistical differences were observed between EM and CTRL women.

For patients with wild-type exon 1 genotype A/A, acute-phase responsiveness was affected by the promoter X/Y polymorphism. The YA haplotype was associated with positive acute-phase responsiveness, while negative acute-phase responsiveness was associated with its absence [49]. One limitation of the study is the inability to define the X/Y promoter polymorphism in EM patients with high MBL levels. This information could be crucial in understanding the correlation between high MBL levels and the severity of the disease.

We tried to uncover the local role of MBL and MASP-2 in the endometriotic lesions. To this end, we investigated the local expression of mRNAs by RT-qPCR and the protein presence by IHC in ectopic and eutopic endometrium. Surprisingly, we failed to detect both transcripts in the lesions, whereas we found an intense staining for MASP-2 in both ectopic and eutopic endometrium. These findings lead to the hypothesis of a local deposition of MASP-2 in the endometrium, likely caused by the activation of the coagulation pathway during the menses bleeding of the ectopic tissue. We can surmise that the presence of MBL in eutopic endometrium, particularly on the cylindrical epithelium, could be important for maintaining uterine microbial equilibrium.

Intrauterine infection and inflammation are known to be major risk factors in the development of gynaecological diseases. However, directly targeting the elimination of infection has not been proven effective to reduce the risk, probably due to additional pathogenic bacterial species that stimulate inflammatory pathways. Some of these bacteria may not be detectable through traditional culture-based methods, may be resistant to standard treatments, or may not be associated with overt clinical infection. Using of 16S rRNA gene sequencing to analyze samples collected from different sites could help in identifying a more significant number of potentially harmful microbial species. In our cohort, we observed a possible crosstalk between the resident microbiome and the activation of the complement pathway. In the endometrium, the defective activation of the LP seemed to render women more susceptible to increased colonization of pathogens [50,51]. Particularly, a non-Lactobacillus dominated uterine microbiome was observed, allowing the overgrowth of pathogens such as *Gardnerella, Prevotella, Atopobium, Streptococcus,* and *Escherichia-Shigella* [52]. Consistent with our observations, Khanl et al. earlier reported that the endometrial microbiome of EM patients is composed of non-lactobacillus *spp.* [53]. In the vaginal microbiome, the LP activation was accompanied by dysbiosis, particularly characterized by the presence of genera such as *Gardnerella* and *Prevotella*. We can speculate that the increase in circulating MBL levels in EM patients (although not statistically significant) could create an inflammatory context in which opportunistic pathogens can thrive, further increase inflammation, and likely ascend to the upper reproductive tract. In this scenario, the number of shared bacteria between the vagina and the endometrium, likely following an ascension from the lower to the upper reproductive tract, is higher than the condition in which the LP activation is low or normal. In this regard, menstrual blood analysis would provide valuable insight into the activation of endometrial cells following vaginal and endometrial dysbiosis. However, in our pilot study, we focused on the potential microbial crosstalk between the vaginal and endometrial environments, given the close anatomical relationship. It is widely recognized that the menstrual cycle phase influences the microbiome and the immune system in these tissue districts [13]. Unfortunately, a limitation of the study is that we were unable to associate these data because out of 38 patients, 23 were taking oral contraceptives. Of the 15 patients who were not treated, we could obtain information on the cycle phase only for 6 women.

Pellis *et al.* showed that MBL levels, analyzed in cervicovaginal lavage taken from women during different phases of their menstrual cycle, were higher during the secretive phase, which comes from both circulation through transudation and local synthesis. The endometrium's contribution is negligible, and the basal layer of the epithelium of vaginal tissue is the source of MBL [54]. Furthermore, this group demonstrated that MBL and C3 present in the vaginal cavity function as recognition molecules for infectious agents that colonize the cervicovaginal mucosa [55].

The microbiome composition can affect the activation or dysregulation of complement pathways in the reproductive tissues and vasculature. Complement dysregulation in the intrauterine environment can exacerbate inflammation and the production of inflammatory mediators, which, in turn, can lead to direct changes in the cervix, such as collagen degradation, and activation of the uterine endometrium [56]. A recent study found *Fusobacterium* in the endometrium and endometrial lesions of over 50 % of EM patients but only 7 % of CTRLs. In murine studies, *Fusobacterium* inoculation increased endometriotic lesion numbers and weight, while metronidazole and chloramphenicol reduced them. These findings suggest that *Fusobacterium* infection may contribute to EM pathogenesis and that anti-microbial treatment to eradicate endometrial infection should be further studied [19]. Another limitation of this study was the lack of an adequate model to support the evidence of the cross-talk between LP and dysbiosis in EM. The only definitive strategy directly correlating LP deficiency with endometrial dysbiosis would involve conducting tests on MBL-deficient mouse models. However, it is important to note that there are two genes for MBL in mice, which complicates the evaluation. Furthermore, findings regarding the microbiota in animal models often lack correlation with human conditions due to numerous influencing factors.

Our genotyping data showed that the difference in MBL

concentrations between the EM and CTRL groups could not be attributed to the MBL polymorphisms; thus, we further assessed potential modulating factors affecting its expression. Given that EM is both a chronic inflammatory and estrogen-dependent disease, we investigated the role of E2 in the modulation of MBL expression by hepatocytes. Normal values for E2 vary depending on the phase of the menstrual cycle. In the follicular phase, 50–70 pg/mL; peak ovulation, 200–400 pg/mL; luteal phase, 100–150 pg/mL [57]. We stimulated HepG2 cells with 20, 100, 350, 500, or 1000 pg/mL of E2. It appears that physiological or suboptimal concentrations of E2 (100 pg/mL) inhibits the expression of MBL by HepG2 cells, while medium-to-high concentrations (350 pg/mL) enhances it. Curiously, at higher concentrations (500–1000 pg/mL), the MBL expression dropped again, with a trend that seems biphasic. It is well known that estrogen-containing oral contraceptives increase the plasma concentration of clotting factors II, VII, X, XII, factor VIII, and fibrinogen [58]. Still, no data about the hepatic synthesis of complement components are available.

To conduct a more detailed analysis of how 17-β estradiol affects MBL expression, we focused our WES data analysis on a specific group of 44 genes associated with the estrogen pathway. These genes are known to have an impact on 17-β estradiol levels. Sixteen percent (6/35) of EM patients were carriers of these rare variants. Of note, five out of these six patients were characterized by MBL deficiency. This observation led to the hypothesis that these rare damaging variants within estrogen signaling-related genes could impact proper gene function, thus influencing 17-β estradiol levels. Indeed, we speculate that the impaired regulation of 17-β estradiol levels could be involved in determining the observed MBL altered levels.

5. Conclusions

In conclusion, our study indicate that, although not statistically significant, the group of women with EM has a higher percentage of individuals with LP deficiency. Additionally, this group shows a broader distribution of circulating MBL concentrations; MBL levels correlate with the severity of the condition. The endometrial dysbiosis seen in patients with EM may be linked to a deficiency in LP functionality, while the altered levels of MBL could result from a dysfunctional estrogen pathway. We acknowledge the limitations related to sample size and the heterogeneity in our study groups regarding age, EM stages, and treatment variations. These limitations stem from the pilot and monocentric nature of the study, which restricted the enrollment to a small number of patients.

Abbreviations

AP	alternative pathway
AEC	3-amino-9-ethylcarbazole
CE	control endometrium
CP	classical pathway
CTRL	control
EM	endometriosis
GEP	gene expression profiling
HRMA	High-Resolution Melting Analysis
IHC	immunohistochemistry
LP	lectin pathway
MAF	minor allele frequency
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
OMA	ovarian endometrioma
PCoA	principal coordinate analysis
PE	patient endometrium
rASRM	revised American Society for Reproductive Medicine
rRNA	ribosomal RNA
RT-qPCR	Real-Time quantitative PCR
WES	whole-exome sequencing

CRediT authorship contribution statement

Miriam Toffoli: Writing – original draft, Methodology, Data curation, Conceptualization. **Giuseppina Campisciano:** Writing – original draft, Methodology, Data curation, Conceptualization. **Aurora Santin:** Writing – original draft, Methodology, Data curation, Conceptualization. **Silvia Pegoraro:** Methodology, Data curation, Conceptualization. **Gabriella Zito:** Resources, Investigation. **Beatrice Spedicati:** Conceptualization. **Andrea Balduit:** Writing – review & editing, Visualization, Data curation. **Federico Romano:** Resources. **Giovanni Di Lorenzo:** Resources. **Alessandro Mangogna:** Resources, Data curation. **Paola Tesolin:** Formal analysis. **Giuseppe Giovanni Nardone:** Formal analysis. **Nunzia Zanotta:** Methodology. **Serena Sanna:** Resources, Investigation. **Francesca Crobu:** Resources. **Uday Kishore:** Writing – review & editing. **Giuseppe Ricci:** Supervision, Resources, Funding acquisition. **Roberta Bulla:** Writing – review & editing, Supervision, Funding acquisition. **Giorgia Girotto:** Writing – review & editing, Supervision, Funding acquisition. **Chiara Agostinis:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

Authors' contributions

CA, MT, GC, AS, SP, and BS designed the study. MT, GC, AS, SP, and NZ performed the experiments. MT, GC, AS, SP, AB, AM, GGN, PT and CA analyzed data. GZ, GDL, FR, AM, SS, FC, and GR provided resources and conceptual advice. CA, AB, AS, GC, MT, UK, RB, and GG wrote and revised the manuscript. CA, GG, GR, and RB supervised the project. CA, GG, GR, and RB provided funding. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2025.123427.

Data availability

The data that support this study's findings are available in the manuscript and in the Supplemental material. The microbioma sequencing data are openly available in the SRA NCBI Archive: BioProject ID PRJNA1179883.

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