Platelets as key cells in endometriosis patients: insights from small extracellular vesicles in peritoneal fluid and endometriotic lesions analysis

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Abstract

Endometriosis is a chronic inflammatory condition characterized by the presence of endometrium-like tissue outside the uterus, primarily affecting pelvic organs and tissues. In this study, we explored platelet activation in endometriosis. We utilized the STRING database to analyze the functional interactions among proteins previously identified in small extracellular vesicles (EVs) isolated from the peritoneal fluid of endometriosis patients and controls. The bioinformatic analysis indicated enriched signaling pathways related to platelet activation, hemostasis, and neutrophil degranulation. Double immunohistochemistry analysis for CD61 and MPO revealed a significant presence of neutrophils and platelets in close contact infiltrating endometriotic lesions, suggesting potential cell-cell interactions. Subsequently, we isolated small EVs from the peritoneal fluid of women diagnosed with endometriosis and from women without endometriosis who underwent surgery for non-inflammatory benign diseases. We performed single-particle phenotyping analysis based on platelet biomarkers GPIIb/IIIa and PF4 using nanoflow cytometry, as well as single-particle morphological and nanomechanical characterization through atomic force microscopy. The study demonstrated that patients with endometriosis had a notably higher proportion of particles testing positive for platelet biomarkers compared to the total number of EVs. This finding implies a potential role for platelets in the pathogenesis of endometriosis. Further research is necessary to delve into the mechanisms underlying this phenomenon and its implications for disease progression.

Keywords: endometriosis, extracellular vesicles, platelet, neutrophil, follicular fluid, peritoneal fluid, GPIIb/IIIa, PF4

1. Introduction

Endometriosis is an estrogen-dependent chronic inflammatory process that affects 10–15% of women of reproductive age [1,2]. The condition is characterized by the presence of endometrium-like tissue outside the uterus, mainly in pelvic tissues, and can result in various types of debilitating pain and subfertility [3]. Previous studies have shown that various alterations in inflammatory cells, complement components, cytokines, and chemokines within endometriotic lesions and peritoneal fluid contribute to the establishment of an inflammatory microenvironment [4–7]. Significantly, this inflammatory niche interacts with endometriotic cells, including stromal and epithelial cells, playing a pivotal role in the development and persistence of endometriosis. For instance, the inflammatory microenvironment can enhance the survival and invasion of endometriotic cells [8] and contribute to the onset of pain and infertility associated with the condition [9,10]. Moreover, the communication between inflammatory cells and endometriotic cells can perpetuate the inflammatory response, creating a harmful cycle that sustains the disease process [11]. Moreover authors have demonstrated a link between the hemostasis system, specifically platelet hyperactivity, and the development and presentation of endometriosis symptoms [12]. Platelets play a role in promoting angiogenesis, inflammation, and fibrosis within endometriosis lesions [13–16]. Fibrosis is a biological process that involves activated platelets, macrophages, and myofibroblasts [17]. Activated platelets in endometriosis lesions produce growth factors, cytokines, and chemokines such as TGF-β1, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and connective tissue growth factor (CTGF), which contribute to fibrogenesis [17]. Research has shown a dynamic interaction between platelets and endometriotic lesions, leading to the development of endometriosis and causing a hypercoagulable state in affected women [13,18]. Overall, a combined effect of the inflammatory response and hypercoagulation status may exist in advanced stages of endometriosis [19,20]. Therefore, targeting the coagulation pathway through anticoagulant or

70 antiplatelet therapy offers a potential treatment option for endometriosis and provides
71 opportunities for identifying innovative biomarkers [12,13].

72 Extracellular vesicles (EVs) are membrane-bound particles that serve as highly efficient
73 carriers of intercellular communication by transporting proteins, lipids, and nucleic acids [21].
74 Studies have demonstrated that EVs play a crucial role in cellular communication in
75 endometriosis by modulating immunosuppressive and proinflammatory pathways [22,23].
76 Exploring EVs in endometriosis has the potential to offer valuable insights into the intricate
77 mechanisms that drive the disease [24,25]. Through cargo analysis, researchers have the potential
78 to discover new biomarkers for early endometriosis detection or identify novel therapeutic
79 targets [26]. Moreover, deciphering how EVs contribute to the inflammatory microenvironment
80 in endometriosis could pave the way for developing targeted treatments that disrupt this
81 communication, potentially halting the disease's progression.

82 Platelet-derived EVs are released during platelet activation [27] and can be measured
83 using a variety of selectively expressed markers [28]. In a previous study, we used single-particle
84 phenotyping analysis based on platelet biomarkers, such as GPIIb/IIIa and PF4, to assess
85 platelet activity in the ascites of women with ovarian cancer [29]. Given the significant role of
86 intraperitoneal fluid in the pathophysiology of endometriosis, as it serves as a dynamic
87 interface between the immunological and reproductive systems [4,5,30,31], our goal was to evaluate
88 platelet activity within this specific environment. Peritoneal fluid samples were collected from
89 women diagnosed with endometriosis for this purpose. We employed the same single-particle
90 phenotyping analysis that had successfully measured platelet activity in ovarian cancer
91 patients.

2. Material and Methods

2.1. Proteome data enrichment and pathway analysis

The proteins of exosomes in peritoneal liquid from endometriosis patients and controls were retrieved from published literature in PubMed [24]. The protein-protein interaction (PPI) network was visualized by the freely accessible, web-based Search Tool for Recurring Instances of Neighboring Genes (STRING) software [32]. In addition to PPI network analysis, functional enrichment analysis using the Reactome database was performed.

2.2. Patient cohort and sample collection

Patients who underwent elective laparoscopy for suspected endometriosis due to pain symptoms and/or infertility were recruited during the preoperative assessment. Following approval by the Institutional Review Board (IRB-BURLO 01/2022, 09.02.2022), patients were asked to sign an informed consent form. The inclusion criteria for endometriosis patients were as follows: i) had a histological confirmation of superficial peritoneal endometriosis (SPE); ii) had a histological confirmation of deep infiltrating endometriosis (DIE); and iii) had a histological confirmation of ovarian endometrioma. The inclusion criteria for control patient selection were patients without endometriosis who underwent surgery for benign, not inflammatory, conditions. The exclusion criteria were as follows: i) interdicted patients who were unable to provide informed consent; ii) patients with other forms of endometriosis without endometriosis according to the inclusion criteria; iii) patients with peritoneal inflammatory diseases (i.e., pelvic inflammatory disease, diverticulosis, etc.); and iv) oncological patients with peritoneal involvement. The follicular fluid (FF) of the first and largest punctured follicle from the bilateral ovaries was collected during the oocyte retrieval procedure (transvaginal follicular aspiration). Each ovarian follicle was aspirated independently. As previously described [29], hemoglobin levels were measured in all the samples to determine the presence of blood contamination. Only uncontaminated samples were included.

117 Peritoneal fluid from 12 patients was used in the present study. The patients'
118 characteristics and medications are reported in **Table 1**. We included 70 patients in the study,
119 11 of whom had free fluid in the pouch of Douglas. Six blood-free samples were used in this
120 investigation. Additionally, follicular fluid from 5 infertile patients who underwent in vitro
121 fertilization was analyzed.
122
123 **Table 1**. Patient characteristics.

Patient	Age	Symptoms/medical history
Endometriosis #1	26	Dysmenorrhoea, catamenial dyschezia. No comorbidities. Stage II ASRM.
Endometriosis #2	41	Dysmenorrhoea, dyspareunia, chronic pelvic pain, catamenial dyschezia. No comorbidities. Stage IV ASRM.
Endometriosis #3	33	Paucisymptomatic. No comorbidities. Stage IV ASRM.
Endometriosis #4	47	Dysmenorrhoea, dyspareunia, chronic pelvic pain, catamenial dyschezia. No comorbidities. Adenomyosis, leyomiomatosis. Stage I ASRM.
Endometriosis #5	28	Dysmenorrhoea, dyspareunia. No comorbidities. Stage II ASRM.
Endometriosis #6	43	Paucisymptomatic. Vitiligo, atopic dermatitis. Leyomiomatosis. Stage II ASRM.
Control #1	44	Leyomiomatosis.
Control #2	37	*BRCA1* mutation. No other comorbidities.
Control #3	48	Leyomiomatosis, adenomyosis., Hashimoto's thyroiditis
Control #4	49	Leyomiomatosis, essential arterial hypertension.
Control #5	57	Leyomiomatosis.
Control #6	32	Leyomiomatosis.

124 Note: The American Society for Reproductive Medicine (ASRM) classification system is divided into four stages
125 or grades according to the number of lesions and depth of infiltration: minimal (Stage I), mild (Stage II), moderate
126 (Stage III) and severe (Stage IV). Abbreviation: *BRCA1*, BReast CAncer gene 1.
127
128

129 *2.3. Small-EV isolation*

130 The isolation of small EVs from peritoneal and follicular fluids was conducted
131 according to a previously established method [33] using Total Exosome Isolation Reagent
132 (Invitrogen, CN 4484453) and according to the manufacturer's provided methodology. Briefly,
133 we centrifuged the samples at 2000 × g to remove cells and debris. Subsequently, we recovered
134 the small EVs from the resulting liquid through precipitation.

135 *2.4. Nanoflow cytometry*

136 The nanoflow cytometry (nFCM) procedure was carried out using a previously
137 established method [29]. Briefly, 10 µl of the EV sample was incubated with $2e^{10}$ particles/ml
138 (PF4: 1:10, GPIIb/IIIa: 1:10, CellMask: 1:500) before incubation at room temperature for 1 h.
139 After incubation, the mixture was diluted in PBS to 1 ml and ultracentrifuged for 1 hour at
140 100,000 × g using a Beckman Coulter benchtop optima TLX. For rapid nFCM analysis, pellets
141 were resuspended to $1e^8$-$1e^9$ particles/ml after supernatant removal. The nFCM Professional
142 Suite v1.8 program processed the data and generated dot plots, histograms, and statistical data
143 in a PDF. Gating inside the program enabled proportional analysis of subpopulations
144 segregated by fluorescent intensities, with size distribution and concentration accessible for
145 each.

146 *2.5. Atomic Force Microscopy (AFM)*

147 AFM-based quantitative morphology was performed as described elsewhere to obtain
148 the size and stiffness distributions of the individual particles found in all the samples [34]. Briefly,
149 5 µl aliquots from EV samples were deposited on poly-L-lysine-functionalized glass coverslips
150 and left to adsorb for 30 min at 4°C. AFM micrographs were recorded in liquid at RT on a
151 Multimode 8 microscope (Bruker, USA) equipped with Scanasyst Fluid+ probes (Bruker,
152 USA) and operated in peak-force mode. Image analysis was performed with Gwyddion v2.61
153 and custom Python scripts [35]. All samples were prepared following the same deposition

154 protocol, thus allowing us to estimate the relative particle concentrations of the samples via
155 their respective particle surface densities, as discussed elsewhere [36].

156 *2.6. Immunohistochemical Analysis*

157 Endometriosis tissue samples were fixed in a 10% v/v buffered formalin solution before
158 being embedded in paraffin (formalin-fixed and paraffin-embedded, FFPE). Tissue sections,
159 cut to a thickness of four micrometers, were then deparaffinized and rehydrated. The antigen
160 unmasking technique was performed, using citrate buffer, pH 6, or Tris-EDTA buffer, pH 9, in
161 thermostatic bath at 98°C for 30 min. Sections were then allowed to reach room temperature
162 and washed in phosphate-buffered saline (PBS). After neutralization of endogenous peroxidase
163 activity with 3% v/v H_2O_2 and blocking of non-specific bindings by PBS + 2% bovine serum
164 albumin, samples were incubated with CD61 primary antibodies overnight at 4°C. Staining
165 was revealed via anti-rabbit HRP-conjugated secondary antibody and 3,3′-diaminobenzidine
166 (DAB, Dako, Agilent, Denmark) substrate chromogen. Slides were then counterstained with
167 Mayer Hematoxylin (Diapath, Italy).

168 Multiplex immunohistochemical staining was performed on FFPE human tissue
169 sections as previously described. Briefly, three-micrometer-thick tissue sections were
170 deparaffined, rehydrated and unmasked using Novocastra Epitope Retrieval Solutions pH9 in
171 a thermostatic bath at 98°C for 30 minutes. The sections were brought to room temperature and
172 washed in PBS. After neutralization of endogenous peroxidase with 3% H_2O_2 and Fc blocking
173 with 0.4% casein in PBS (Leica Biosystems), the sections were incubated with the following
174 primary antibodies: mouse monoclonal CD61 (ready to use, clone 2F2 cod. PA0308, Leica
175 Biosystems) and rabbit polyclonal MPO (myeloperoxidase) (1:25, cod. ab9535, Abcam).
176 Double IHC staining was performed by applying SignalStain® Boost IHC Detection mouse,
177 horseradish peroxidase (HRP) conjugated produced in goat (cod. 8125S, Cell Signaling
178 Technology) and SignalStain® Boost IHC Detection rabbit, alkaline phosphatase (AP)-

179 conjugated produced in horse (cod. 18653S, Cell Signaling Technology) and DAB (3,3′-
180 Diaminobenzidine, Leica Biosystems) or Vulcan Fast Red (BioOptica) as substrate
181 chromogens. All the sections were analyzed under Zeiss Axio Scope A1, and
182 microphotographs were acquired using Axiocam 503 Color digital camera with the ZEN2
183 imaging software.
184

185 ## 3. Results

186 Nazry *et al*. identified a set of unique proteins derived from exosomes isolated from the
187 peritoneal fluids of endometriosis patients and controls [24]. We employed bioinformatic data
188 analysis to characterize signaling dynamics, and the PPI network analysis findings confirmed
189 extensive and intricate interactions within the unique protein lists. **Figure 1** depicts the
190 predicted PPI network during the proliferative phase of control and endometriosis stages I and
191 II. Nodes represent proteins, edges indicate functional and physical protein associations, line
192 thickness reflects the strength of data support, and only interactions with a high confidence
193 score of ≥ 0.7 are visualized. The pathways of the innate immune system, neutrophil
194 degranulation, platelet degranulation, hemostasis, and formation of the cornified envelope were
195 identified as the top pathways that comprised an interconnected set of proteins in all of the
196 samples analyzed (**Supplementary 1** shows the analysis of controls and endometriosis stage
197 I/II in the secretive phase).

198 Subsequently, we isolated small EVs from the peritoneal fluid of women diagnosed
199 with endometriosis and from women without endometriosis who underwent surgery for non-
200 inflammatory benign diseases. **Figure 2** illustrates the workflow of the analyses performed on
201 the samples.
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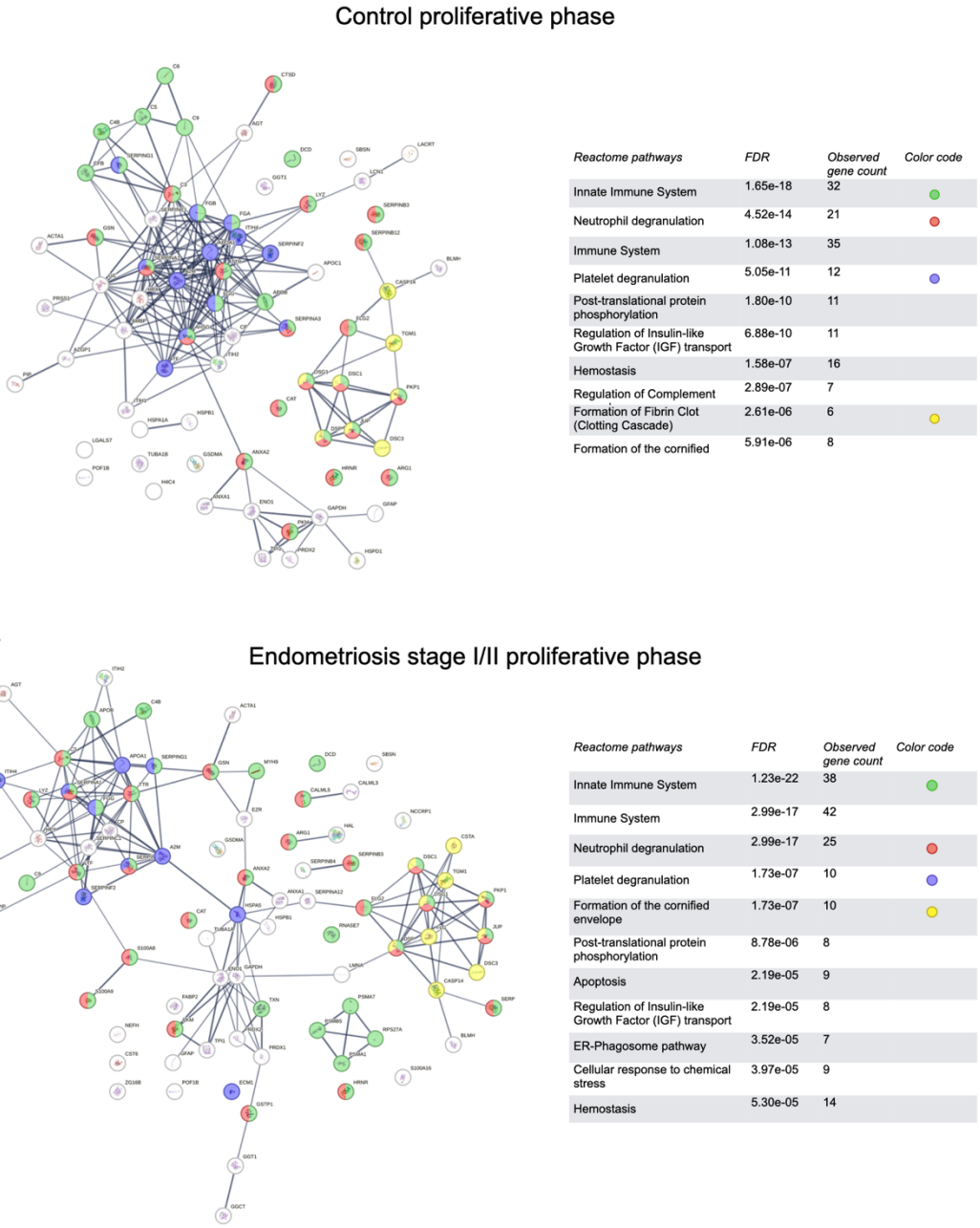

Figure 1. STRING protein-protein interaction (PPI) network analysis of proteins found in exosomes isolated from the peritoneal fluid during the proliferative phase in control patients (upper panel) and endometriosis patients (lower panel). In this network, nodes represent proteins, edges indicate functional and physical protein associations, and the thickness of the lines reflects the strength of data support. Only interactions with a high confidence score of ≥ 0.7 are displayed. The functional pathways were obtained from the Reactome database, and the top 10 pathways are listed in ascending order based on their *p*-value. FDR: False Discovery Rate.

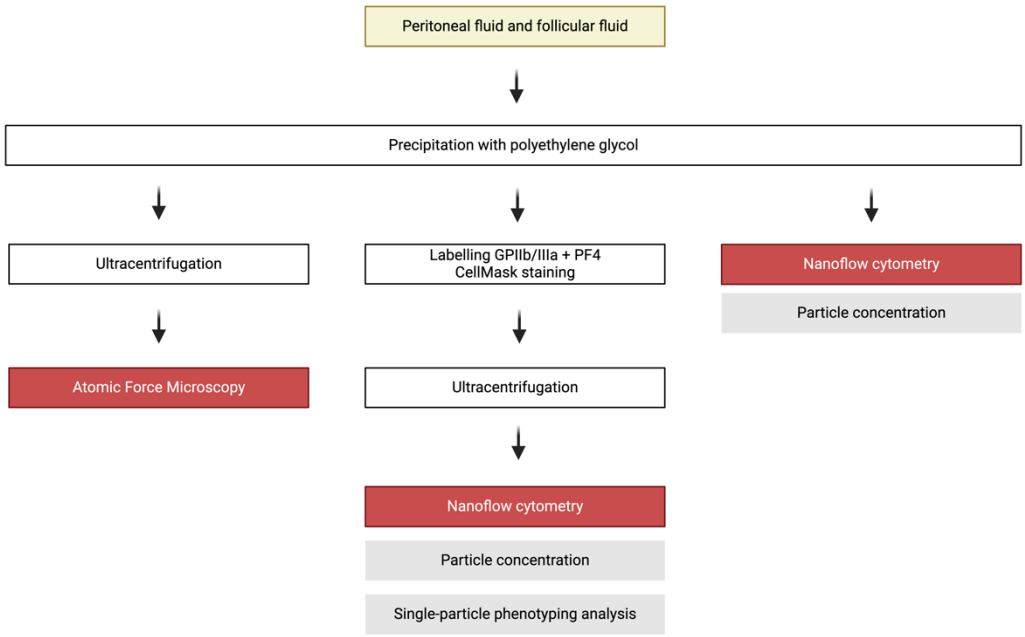

Figure 2. Overview of the analytical workflow.

The precipitation isolated samples contained approximately 10^9 to 10^{11} particles/ml, measured via FCM, which decreased to 10^8-10^9 after UC. Both nFCM and AFM agreed to assign the highest particle concentrations to follicular fluid samples after UC. However, these follicular fluid-isolated particles showed low proportions of Cell Mask Deep Red (CMDR) labeling, typically below 20%. Little difference was observed between the control and endometriosis particle concentrations post-UC and the CMDR labeling, which, on average, accounted for 40% of the particles in these samples. The characteristics of the particle concentration, size, and percentage of membrane-positive particles in the samples measured via nFCM are shown in **Figure 3**. The combined morphological and nanomechanical characteristics of the individual particles in the selected samples were measured via AFM, confirming abundant EVs and residual content of non-vesicular particles with diameters less than 30 nm (**Figure 4**). Particles displaying nanomechanical behavior previously associated with intact vesicles [34,36] were found to have an average contact angle of ~90°, which

228 corresponds to a mechanical stiffness largely in line with values previously measured with the

229 same technique on, *e.g.,* samples isolated from ascites [29].

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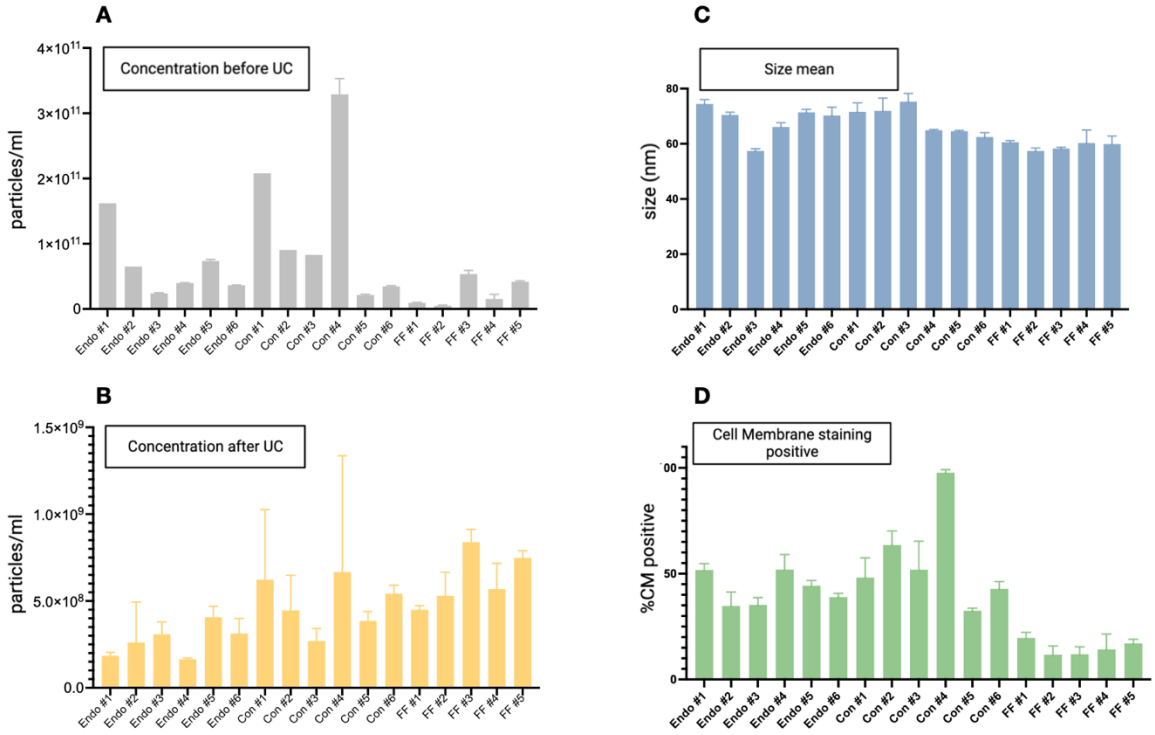

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232 **Figure 3.** Nanoflow cytometry analysis (NanoFCM) of small EVs from peritoneal and follicular fluids in a cohort

233 of patients. The data are reported as the means ± SDs of one experiment performed in triplicate. (**A**) The

234 concentration of all particles > 45 nm in diameter in precipitation-isolated samples. (**B**) The concentration of all

235 particles > 45 nm following ultracentrifugation (UC). (**C**) Size following UC. (**D**) The percentage of all particles

236 > 45 nm, which also showed CellMask™ labeling (thus determined as EVs). Abbreviations: Endo, endometriosis

237 sample; Con, control sample; FF, follicular fluid sample.

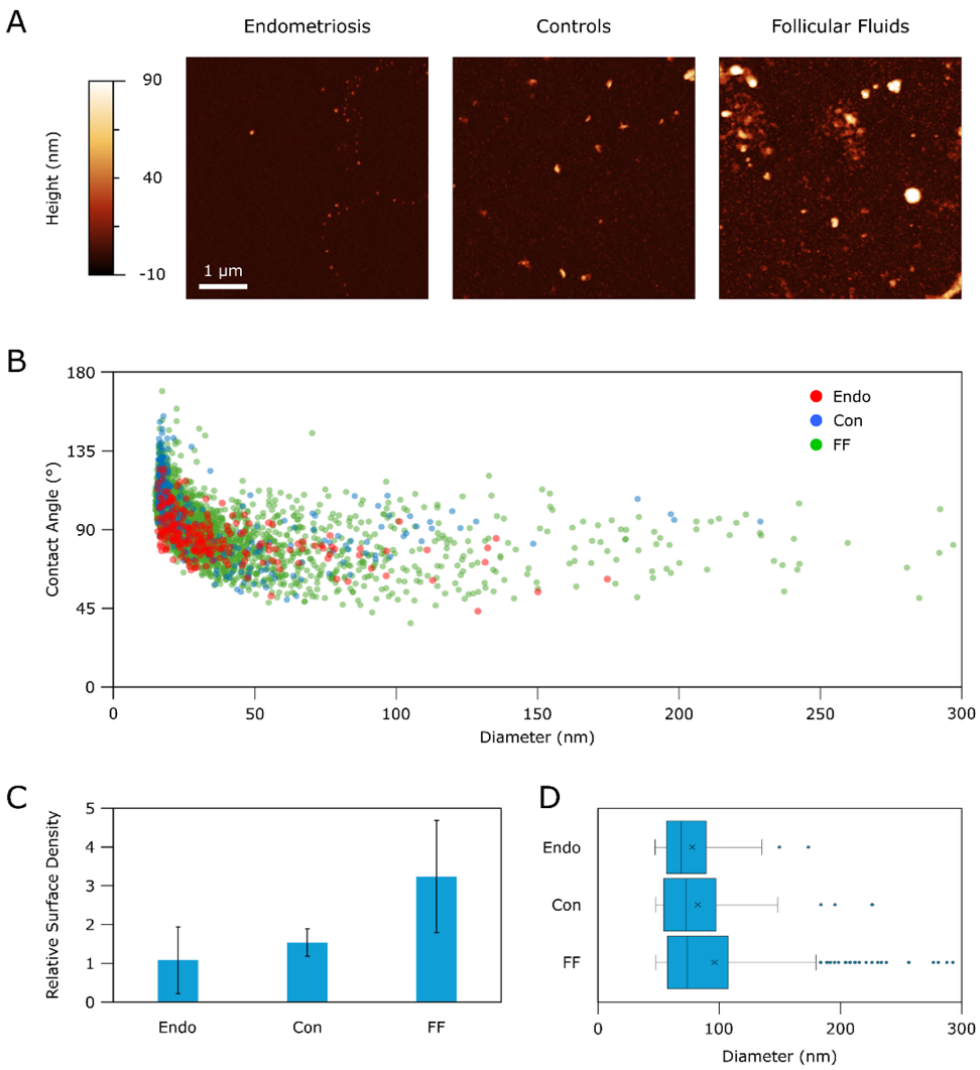

Figure 4. Atomic force microscopy (AFM)-based morphometry and nanomechanical screening. (**A**) Representative AFM micrographs of the three sample types. (**B**) Contact angle (CA) *vs* diameter plot of individual particles measured from at least 10 AFM micrographs deposited from duplicate preparations (Endo: N=227; Con: N=296; FF: N=3632). The contact angle can be regarded as a measure of the mechanical stiffness of EVs; all particles above ~45 nm display the typical conserved average CA of intact vesicles, whereas smaller particles do not. (**C**) Normalized relative surface density of particles with diameters greater than 45 nm, showing that the FF samples are consistently more concentrated than the Endo and Con samples. (**D**) Box plot of the measured diameters of individual vesicles greater than 45 nm shows how FF samples contained the most significant proportion of larger EVs, while Endo samples contained the smallest. Abbreviations: Endo, endometriosis sample; Con, control sample; FF, follicular fluid sample.

250 Single-particle phenotyping analysis enabled the measurement of the platelet-
251 associated markers GPIIa/IIIb and PF4 in all the samples, as well as EV membrane staining
252 (**Figure 5**). The percentages of small-EVs that were positive for platelet markers in follicular
253 fluids and control fluids were less than 2.9% and 3.7%, respectively, with no statistically
254 significant differences between these two sets of samples. Endometriosis patients' samples had
255 higher levels, ranging from 3.4% to 17%, with a statistically significant difference between
256 these samples and follicular fluid and control samples.
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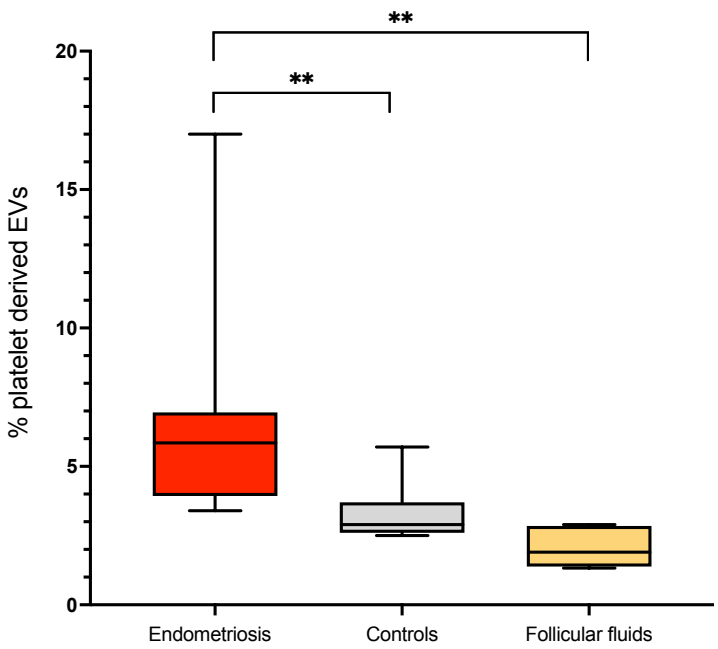

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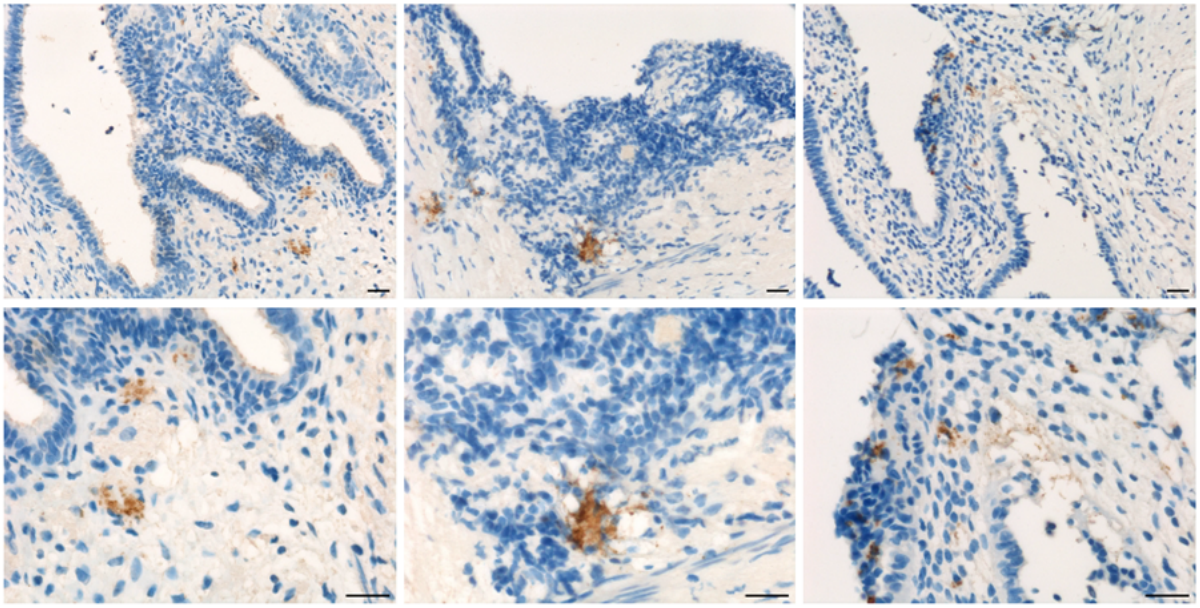
259 **Figure 5.** Percentage of particles positive for GPIIb/IIIa and PF4 relative to total EVs from peritoneal fluid in
260 women with endometriosis (endometriosis), in women without endometriosis during surgery for a benign, non-
261 inflammatory, condition (controls), and in follicular fluid samples collected from infertile patients undergoing in
262 vitro fertilization (follicular fluids). The samples were fluorescently labeled with FITC-conjugated antibodies
263 specific for GPIIb/IIIa and PF4. Additionally, the dye CellMask™ deep red plasma membrane stain (CM) was
264 used to distinguish EVs from other small particles.
265

266 We performed immunohistochemistry (IHC) for CD61 on FFPE biopsies from patients with
267 endometriosis to examine the presence and distribution of platelets. As shown in **Figure 6**,
268 CD61-positive platelet clusters were observed surrounding the endometriotic lesions.
269



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271 **Figure 6**. Microphotographs showing platelets in endometriotic lesions. Representative immunohistochemical
272 staining of CD61. DAB (brown) chromogen was used to visualize the binding of the anti-human CD61 antibody.
273 Magnifications were 200x (up), and 400x (down); scale bar, 50 µm.
274

275 We then investigated platelet activation within endometrioid lesions, focusing on their
276 interaction with inflammatory cells using a multiplex immunolocalization assay for CD61 and
277 MPO. Our analysis revealed both tissue and intravascular deposits of platelets within the
278 lesions, with CD61-positive platelets closely associated with MPO-expressing granulocytes.
279 This suggests a potential cell-cell interaction between neutrophils and platelets infiltrating the
280 lesions (**Figure 7**).
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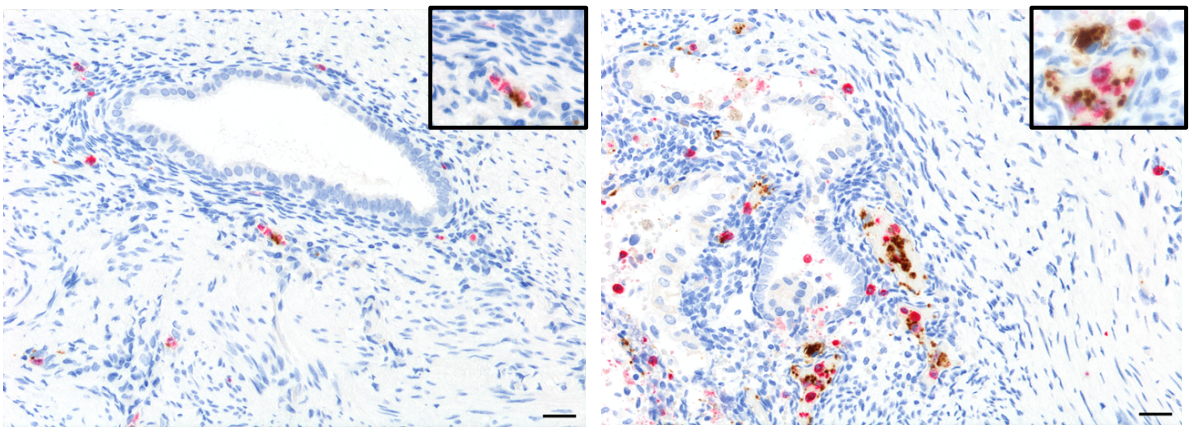

Figure 7. Representative microphotographs of double immunohistochemical staining for CD61 and MPO in endometriotic foci highlighting a close-contact between platelet aggregates (CD61+) and neutrophils (MPO+) in the cytogenic stroma of endometriotic lesions. As shown in inset at the bottom of the pictures, this interaction occurs at tissutal and intravascular sites. DAB (brown) chromogen was used to visualize the binding of the anti-human CD61 antibody, while AEC (red) chromogen was used to visualize the binding of the anti-human MPO antibody. The images were captured at magnifications of 200x (top) and 400x (bottom); scale bar, 50 µm.

4. Discussion

Endometriosis is considered a chronic inflammatory condition, and the primary approach to medical treatment involves the use of hormonal and anti-inflammatory medication [2,37]. Scientific data shows a strong association between the coagulation system and the progression of endometriosis [12,13,18,19]. Recent study data has shown the role of platelets in the progression of endometriosis, which supports the use of anticoagulants as a treatment for endometriosis. Additionally, it emphasizes the significance of evaluating platelet activation in individuals with endometriosis. Together with myofibroblasts, macrophages, and sensory nerve fibers, platelets are among the main cellular elements implicated in the pathophysiology of fibrosis [17].

Consistent with the literature [13], this research has found that platelet agglomerates in the cytogenic stroma of lesions suggest their migration from the microenvironment into the

312 lesions. It was shown that platelets play essential roles in the development of endometriosis
313 [13,14,38–40] and it was found that endometriotic stromal cells release platelet-activating molecules
314 [41]. We also demonstrated a possible interaction between platelets and neutrophils in
315 endometriosis foci. Platelets release signals that attract neutrophils to sites of inflammation.
316 For example, they secrete chemokines like CXCL4 (PF4) and CXCL7 that recruit neutrophils
317 [42]. Platelets express P-selectin on their surface upon activation, which binds to PSGL-1 on
318 neutrophils, facilitating their interaction [43,44]. Platelets can induce the formation of neutrophil
319 extracellular traps (NETs), which could play a role in the pathophysiological mechanisms of
320 endometriosis [45]. Both platelets and neutrophils release pro-inflammatory cytokines that
321 amplify inflammatory responses.

322 To further understand this occurrence, we conducted a series of analyses to determine
323 the presence of platelet activation in the peritoneal fluid of endometriosis patients. The
324 bioinformatic analysis of proteins detected in exosomes of the intraperitoneal fluid has revealed
325 specific biochemical pathways, such as those associated with the immune system, hemostasis,
326 and platelet activation, emphasizing their relevance to the study.

327 The presence of free intraperitoneal fluid collected inside the Pouch of Douglas was
328 consistent with previous literature [46]. In individuals with endometriosis, free fluid was detected
329 in 16% of the patients. A considerable number of samples (45%) were contaminated with blood
330 and were thus excluded from this investigation. The single-particle phenotyping investigation
331 on small EVs indicated the presence of platelet-derived GPIIb/IIIa and PF4 in all tested
332 samples, which is consistent with the bioinformatic analysis highlighting platelet degranulation
333 amongst the enriched pathways in this microenvironment. On the other hand, the assessment
334 of the ratio of this particular EV subset revealed significantly increased values in individuals
335 who had been diagnosed with endometriosis.

The release of follicular fluid during ovulation seems to influence the composition of the peritoneal fluid [47]. Moreover, clinical and experimental data suggest platelets regulate hypothalamic–pituitary–ovarian axis functions [48]. To evaluate the impact of the ovulatory phase on our findings, we quantified the proportion of platelet-derived small EVs in this biological fluid. The isolation process from follicular fluids leads to the production of a population of small EVs. The percentage of platelet-derived EVs in the investigated samples was less than 3%, a substantially lower amount than that observed in the peritoneal fluids of endometriosis patients. Nevertheless, this observed proportion was similar to the levels identified in the control samples. Thus, despite acknowledging the potential influence of follicular fluid on peritoneal fluid composition, the results of this initial investigation suggest no significant impact on the extent of the platelet-derived subpopulation of small EVs.

5. Conclusion

Platelet activation assessment in the microenvironment of endometriosis represents an essential tool for clinical investigations that aim to understand the role of platelets in this pathology and how their inhibition can have a therapeutic effect. The pilot study findings indicated an increase in platelet activity in the peritoneal fluid of patients diagnosed with endometriosis, and emphasize the potential of single-particle phenotyping analysis of the small EVs based on platelet biomarkers. Additional studies are essential to elucidate the precise contribution of platelet activity to endometriosis's pathogenesis and assess its viability as a biomarker for disease diagnosis and monitoring.

Funding

This work was supported by the Italian Ministry of Health, through the contribution given to the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy.

Author contributions

BB: Conceptualization, Investigation, Methodology, Formal Analysis. RDF: Methodology, Formal Analysis. SM: Methodology, Formal Analysis. BP: Methodology, Formal analysis. RL: Methodology, Formal analysis. FV: Investigation, Methodology, Formal Analysis. MB: Investigation, Methodology, Formal Analysis. AM: Methodology, Formal Analysis. GDL, FR, GZ, FZ, GR: Provision of study materials and patients. VC: Methodology, Formal Analysis. BB: Conceptualization, Investigation, Methodology, Formal Analysis. SB: Conceptualization, Investigation, Methodology, Formal Analysis, Supervision, Writing – original draft and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Competing interests

B.P. and R.L. are employees of NanoFCM and their contributions to this article were made as part of their employment. The authors declare no conflict of interest.

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Review Board (IRB-BURLO 01/2022, 09.02.2022) and patients were asked to sign an informed consent form.

Data Availability

The data supporting the findings of this study are available in the open dissemination research data repository Zenodo with the digital object identifier (DOI): 10.5281/zenodo.10849687.

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