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„Significance of the molecular profile of extracellular vesicles from plasma and peritoneal fluid in the development of endometriosis”

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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To my Parents and my Family

- you have been my lighthouse in every storm

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ABBREVIATIONS

ACN	Acetonitrile
AGC	Automatic gain control
ALIX	Apoptosis-linked gene 2-interacting protein X
APS	Ammonium persulfate
ARCN1	Archain 1
AUC	Area under the curve
BCA	Bicinchoninic acid assay
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA-125	Cancer antigen 125
CALD1	Caldesmon 1
CBX3	Chromobox protein homolog 3
CD	Cluster of differentiation
CD227	Mucin 1
CD-EV(s)	Cell-derived extracellular vesicle(s)
CLTCL1	Clathrin heavy chain-like 1
CMDR	CellMask™ Deep Red
COX-2	Cyclooxygenase-2
COL1A1	Collagen type I alpha 1 chain
COL1A2	Collagen type I alpha 2 chain
Cryo-EM	Cryogenic electron microscopy
DDX41	DEAD-box helicase 41
DDX47	DEAD-box helicase 47
DEP(s)	Differentially expressed protein(s)
DNMT1	DNA methyltransferase 1
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ESCRT	Endosomal sorting complex required for transport

EVs	Extracellular vesicles
FA	Formic acid
FAT3	FAT atypical cadherin 3
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FGL2	Fibrinogen-like protein 2
F-NTA	Fluorescent nanoparticle tracking analysis
FTL	Ferritin light chain
GO	Gene Ontology
HIC2	HIC ZBTB transcriptional repressor 2
HIF-1α	Hypoxia-inducible factor 1 alpha
HLA	Human leukocyte antigen
HLA-ABC	Human leukocyte antigen class I A, B, C
HLA-DR	Human leukocyte antigen-DR
HRP	Horseradish peroxidase
ICAM1	Intercellular adhesion molecule 1
IFCM	Imaging flow cytometry
IL	Interleukin
IS	Internal standard
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid chromatography–mass spectrometry
MCP-1	Monocyte chemoattractant protein-1
MESF	Molecules of equivalent soluble fluorophore
MISEV2023	Minimal Information for Studies of Extracellular Vesicles
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
MRPL3	Mitochondrial ribosomal protein L3
mRNA	Messenger RNA
MS	Mass spectrometry
MS2	Tandem mass spectrometry
MUC1	Mucin 1

MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
MUC16	Mucin 16
MVBs	Multivesicular bodies
NGF	Nerve growth factor
NK cells	Natural killer cells
NP-40	Nonidet P-40
NTA	Nanoparticle tracking analysis
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
PF	Peritoneal fluid
PGE2	Prostaglandin E2
PIK3C2A	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha
PPI	Protein–protein interaction
PROCR	Protein C receptor
P/S	Penicillin/streptomycin
RT	Room temperature
S100A8	S100 calcium-binding protein A8
S100A9	S100 calcium-binding protein A9
S100A12	S100 calcium-binding protein A12
SDC4	Syndecan 4
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TARDBP	TAR DNA-binding protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TEAB	Triethylammonium bicarbonate
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th	T helper cells

TIMPs	Tissue inhibitors of metalloproteinases
TMT	Tandem mass tag
TNF-α	Tumour necrosis factor alpha
Tregs	Regulatory T cells
TSG101	Tumour susceptibility gene 101
UPLC	Ultra-performance liquid chromatography
VCAN	Versican
VEGF	Vascular endothelial growth factor
VPS4A	Vacuolar protein sorting-associated protein 4A
VPS4B	Vacuolar protein sorting-associated protein 4B

ABSTRACT

Endometriosis is a chronic, estrogen-dependent gynaecological disease characterized by ectopic growth of endometrial-like tissue and associated with chronic inflammation, immune dysregulation, and extensive tissue remodelling. Despite significant advances in research, its diagnosis still relies primarily on invasive procedures, highlighting the urgent need for reliable, non-invasive biomarkers.

This doctoral thesis aimed to comprehensively characterize and directly compare the proteomic profiles of extracellular vesicles (EVs) derived from peritoneal fluid (PF) and plasma of patients with endometriosis and controls, in order to elucidate both local and systemic molecular mechanisms of the disease and to identify potentially diagnostic biomarker. EVs were isolated using size-exclusion chromatography (SEC) and rigorously characterized in accordance with the Minimal Information for Studies of Extracellular Vesicles 2023 guidelines (MISEV2023). High-resolution, quantitative proteomics based on Tandem Mass Tags (TMT), combined with imaging flow cytometry (IFCM), enabled multidimensional and cross-platform analysis of EV cargo and surface phenotypes.

Proteomic analysis identified over 3,500 proteins in PF-derived EVs and 2,600 proteins in plasma-derived EVs, including a subset of differentially expressed proteins (DEPs) distinguishing endometriosis patients from controls. PF-derived EVs demonstrated a markedly stronger discriminatory capacity, reflecting localized pathological processes within the peritoneal microenvironment, including immune activation, oxidative stress, iron metabolism, extracellular matrix (ECM) remodelling, and cellular adhesion. In contrast, plasma-derived EVs captured more subtle yet consistent systemic alterations associated with immune modulation, vesicle trafficking, and epithelial remodelling.

Importantly, a shared subset of EV-associated proteins was consistently detected across both PF and plasma, revealing a common molecular signature linking local disease activity with its systemic manifestations. Key proteins included inflammatory mediators (e.g., S100 family), ECM components (e.g., COL1A1/2, VCAN), and adhesion-related molecules (e.g., SDC4), suggesting coordinated and multi-compartmental regulation of endometriosis-related processes.

Integration of proteomic profiling with IFCM enabled cross-platform validation of selected EV markers, including CD81 and MUC1 in plasma and HLA-DR in PF, supporting their robustness and translational potential. Furthermore, dynamic changes in EV-associated

markers following surgical treatment indicate their potential utility for monitoring disease activity.

Overall, this study demonstrates that EVs serve as biologically informative carriers of disease-associated signals and, importantly, provide a unique interface between local and systemic aspects of endometriosis. The applied integrative analytical strategy establishes a comprehensive framework for EV-based biomarker discovery and highlights EV-associated proteins as promising targets for the development of non-invasive diagnostic approaches.

STRESZCZENIE

Endometrioza jest przewlekłą, zależną od estrogenów chorobą ginekologiczną, charakteryzującą się obecnością tkanki endometrium poza jamą macicy oraz towarzyszącym jej przewlekłym stanem zapalnym, zaburzeniami odpowiedzi immunologicznej i intensywną przebudową tkanek. Pomimo znacznego postępu badań, diagnostyka choroby nadal opiera się głównie na metodach inwazyjnych, co podkreśla pilną potrzebę identyfikacji wiarygodnych, nieinwazyjnych biomarkerów.

Celem niniejszej pracy doktorskiej była kompleksowa charakterystyka oraz bezpośrednie porównanie profili proteomicznych pęcherzyków zewnątrzkomórkowych (EVs) izolowanych z płynu otrzewnowego (PF) i osocza pacjentek z endometriozą oraz osób zdrowych, w celu uchwycenia zarówno lokalnych, jak i systemowych mechanizmów molekularnych choroby oraz identyfikacji potencjalnych biomarkerów diagnostycznych. EVs izolowano metodą chromatografii wykluczania wielkościowego (SEC), a ich charakterystykę przeprowadzono zgodnie z wytycznymi Minimal Information for Studies of Extracellular Vesicles (MISEV2023). Zastosowanie wysokorozdzielczej proteomiki ilościowej opartej na znakowaniu tandemowymi znacznikami masowymi (TMT) w połączeniu z obrazową cytometrią przepływową (IFCM) umożliwiło wielowymiarową i międzyplatformową charakterystykę zawartości oraz fenotypu powierzchniowego EVs.

Analiza proteomiczna pozwoliła na identyfikację ponad 3500 białek w EVs z PF oraz około 2600 w EVs z osocza, w tym białka różnicująco eksprymowane (DEPs), odróżniające pacjentki z endometriozą od grupy kontrolnej. EVs z PF wykazywały wyraźnie większą zdolność różnicowania odzwierciedlając lokalne procesy patologiczne zachodzące w środowisku otrzewnowym, takie jak aktywacja odpowiedzi immunologicznej, stres oksydacyjny, zaburzenia metabolizmu żelaza, przebudowa macierzy zewnątrzkomórkowej oraz adhezja komórkowa. Z kolei EVs z osocza odzwierciedlały bardziej subtelne, lecz powtarzalne zmiany o charakterze ogólnoustrojowym, związane m.in. z modulacją odpowiedzi immunologicznej, transportem pęcherzykowym oraz przebudową nabłonka.

Co istotne, zidentyfikowano wspólną pulę białek obecnych w EVs zarówno z PF, jak i osocza, co wskazuje na istnienie wspólnego profilu molekularnego łączącego lokalne procesy chorobowe z manifestacją systemową choroby. Do kluczowych białek należały mediatory zapalne (np. rodzina S100), składniki macierzy zewnątrzkomórkowej (np. COL1A1/2, VCAN) oraz cząsteczki związane z adhezją komórkową (np. SDC4), co

sugeruje skoordynowaną regulację procesów związanych z endometriozą w różnych przestrzeniach ustrojowych.

Integracja analizy proteomicznej z IFCM umożliwiła walidację wybranych markerów EVs na poziomie pojedynczych pęcherzyków, w tym CD81 i MUC1 w osoczu oraz HLA-DR w PF, potwierdzając ich stabilność i potencjał translacyjny. Ponadto zaobserwowano dynamiczne zmiany w profilach EV po leczeniu chirurgicznym, co wskazuje na ich potencjalne zastosowanie w monitorowaniu aktywności choroby.

Podsumowując, wykazano, że EVs stanowią biologicznie istotne nośniki sygnałów molekularnych związanych z endometriozą oraz pełnią rolę pomostu między lokalnymi a ogólnoustrojowymi aspektami choroby. Zastosowane zintegrowane podejście analityczne tworzy kompleksowe ramy dla badań nad biomarkerami opartymi na EVs i wskazuje białka związane z EVs jako obiecujące cele do rozwoju nieinwazyjnych metod diagnostycznych.

1. INTRODUCTION

1.1. Endometriosis as a clinical and biological challenge

Endometriosis is a chronic, estrogen-dependent inflammatory disorder characterized by the presence of endometrial-like tissue outside the uterine cavity[1-4]. These ectopic lesions most frequently occur within the pelvic cavity, particularly on the ovaries, pelvic peritoneum, uterosacral ligaments, and rectovaginal septum[1, 2]. However, endometriotic lesions have also been identified in extra-pelvic locations, including the gastrointestinal tract, urinary bladder, diaphragm, lungs, and, in rare cases, even the central nervous system[2, 3]. The ability of endometrial-like cells to establish and persist in ectopic sites reflects the complex biological mechanisms underlying the disease[1, 4].

Endometriosis is considered one of the most prevalent gynaecological disorders affecting women of reproductive age[1, 4]. Epidemiological studies estimate that approximately 10% of women worldwide are affected by the disease, although the true prevalence may be even higher due to underdiagnosis[1, 4]. Among women experiencing infertility, endometriosis is detected in approximately 30-50% of cases, highlighting the strong association between the disease and impaired reproductive function[5]. Despite its high prevalence and significant clinical impact, endometriosis remains poorly understood and frequently underdiagnosed[1, 4].

The clinical presentation of endometriosis is highly heterogeneous[2, 6]. The most commonly reported symptoms include chronic pelvic pain, dysmenorrhea, dyspareunia, dyschezia, and infertility[2, 6]. Additional manifestations such as fatigue, gastrointestinal disturbances, or urinary symptoms may also occur[6]. Interestingly, numerous clinical observations have demonstrated a weak correlation between the anatomical extent of endometriotic lesions and the severity of symptoms experienced by patients[6, 7]. Some individuals with minimal disease may experience severe pelvic pain, whereas others with extensive lesions remain relatively asymptomatic[6]. This discrepancy suggests that factors beyond the mere presence of ectopic lesions contribute to symptom development[7].

Pain associated with endometriosis arises from a combination of inflammatory, neurogenic, and hormonal mechanisms[7-9]. Endometriotic lesions produce various inflammatory mediators, including prostaglandins, cytokines, and growth factors that stimulate nerve fibre growth and increase nociceptive signalling[8, 10, 11]. For example, elevated levels of prostaglandin E2 (PGE2), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and nerve growth factor (NGF) have been detected within the lesion microenvironment[8, 10].

These mediators promote neuroangiogenesis, a process involving coordinated growth of blood vessels and nerve fibres, which contributes to heightened pain sensitivity[7, 8].

Infertility represents another major clinical consequence of endometriosis[5, 12]. Several mechanisms have been proposed to explain the association between the disease and reduced fertility. These include anatomical distortion of pelvic structures, impaired tubal motility, inflammatory alterations in the peritoneal environment, and reduced endometrial receptivity[5, 12]. Inflammatory mediators present in the peritoneal cavity may negatively affect oocyte quality, sperm function, fertilization processes, and embryo implantation[12]. Additionally, alterations in endometrial gene expression and progesterone responsiveness may further impair implantation processes[12].

Beyond its physical manifestations, endometriosis significantly affects patients' quality of life[13]. Chronic pain can interfere with daily activities, professional responsibilities, and social relationships. Many patients report decreased productivity, frequent absenteeism from work, and limitations in maintaining normal routines[13]. Psychological consequences such as anxiety, depression, and emotional distress are also commonly reported[13]. From a broader perspective, endometriosis represents a considerable socioeconomic burden[13, 14]. Healthcare systems incur substantial costs associated with repeated diagnostic procedures, long-term medical treatment, and surgical interventions[14]. Indirect costs related to reduced productivity and loss of working hours further amplify the societal impact of the disease[13, 14].

One of the most challenging aspects of endometriosis management is the prolonged delay between symptom onset and definitive diagnosis[4, 13]. Numerous studies report diagnostic delays ranging from seven to ten years[13]. Several factors contribute to this delay, including symptom variability, lack of disease awareness, and the absence of reliable non-invasive diagnostic biomarkers[4, 13].

Currently, laparoscopic visualization combined with histological confirmation remains the gold standard for diagnosing endometriosis[15]. During laparoscopy, lesions can be directly visualized and biopsied for histopathological evaluation. Although this approach provides a definitive diagnosis, it is inherently invasive and requires surgical intervention[15]. As a result, laparoscopy cannot be used as a screening method and is typically reserved for patients with persistent symptoms. Non-invasive imaging methods such as transvaginal ultrasound and magnetic resonance imaging have improved significantly in recent decades and are valuable for detecting certain forms of endometriosis, particularly ovarian endometriomas and deep

infiltrating lesions[15]. However, superficial peritoneal lesions remain difficult to detect using imaging alone.

The limitations of current diagnostic strategies highlight the urgent need for reliable biomarkers that could facilitate earlier detection of endometriosis[4, 16]. Ideally, such biomarkers should be detectable in easily accessible biological fluids and should reflect molecular processes occurring within endometriotic lesions. Recent advances in molecular biology and omics technologies have opened new opportunities for investigating the biological mechanisms underlying endometriosis[4]. Increasing attention has been directed toward mechanisms of intercellular communication within the lesion microenvironment. Cells within the peritoneal cavity continuously exchange molecular signals that regulate inflammation, angiogenesis, immune responses, and tissue remodelling[4, 9].

Among the various mediators of intercellular communication, extracellular vesicles (EVs) have emerged as particularly important signalling particles[17-19]. EVs are membrane-bound vesicles capable of transporting proteins, lipids, and nucleic acids between cells[17]. Because EV cargo reflects the molecular state of their cells of origin and because these vesicles are present in many biological fluids, they represent promising candidates for biomarker discovery[17-19].

Understanding how EV-mediated communication contributes to the development of endometriosis may therefore provide important insights into disease mechanisms and support the development of novel diagnostic strategies.

1.2. Pathophysiology of endometriosis

Despite decades of intensive research, the precise mechanisms responsible for the development of endometriosis remain incompletely understood. The disease is widely recognized as multifactorial and involves complex interactions between hormonal dysregulation, immune dysfunction, genetic susceptibility, and environmental influences[4, 9, 20, 21]. Rather than being explained by a single pathogenic mechanism, endometriosis is currently considered the result of multiple biological processes that collectively promote the survival, implantation, and growth of endometrial-like tissue outside the uterine cavity[4, 9].

Several complementary theories have been proposed to explain how endometrial cells establish ectopic lesions. These include retrograde menstruation, coelomic metaplasia, stem cell-mediated dissemination, and lymphatic or hematogenous spread of endometrial cells[9, 22]. Although none of these hypotheses alone fully accounts for all clinical observations, together

they provide a conceptual framework for understanding the complex pathophysiology of the disease[9].

In addition to the initial dissemination of endometrial cells, successful lesion formation requires the activation of several biological processes, including cellular adhesion, immune evasion, angiogenesis, extracellular matrix remodelling (EMC), and sustained inflammatory signalling[2, 23]. These mechanisms enable ectopic endometrial cells to survive within the peritoneal cavity and establish persistent lesions.

Importantly, the peritoneal environment in women with endometriosis is characterized by a chronic inflammatory state that promotes the proliferation and invasion of ectopic cells[11, 23, 24]. Elevated levels of inflammatory mediators, including several interleukins (IL-1β, IL-6, and IL-8), TNF-α, and PGE2, have been detected in peritoneal fluid (PF)[11, 24]. These molecules not only stimulate inflammatory responses but also promote angiogenesis and ECM degradation, processes that are essential for lesion establishment[2, 23].

In addition, alterations in hormonal signalling pathways play a central role in the pathophysiology of endometriosis. The disease is characterized by estrogen dominance combined with reduced responsiveness to progesterone, often referred to as progesterone resistance[2, 25]. This hormonal imbalance contributes to increased proliferation of endometrial cells, enhanced inflammatory signalling, and reduced apoptosis of ectopic tissue[2, 25].

Another critical aspect of disease development involves the interaction between ectopic endometrial cells and the surrounding peritoneal microenvironment. Immune cells, stromal cells, endothelial cells, and nerve fibres all participate in a complex network of signalling interactions that regulate lesion growth and maintenance[2, 23, 26]. Through the exchange of cytokines, growth factors, and EVs, these cells create a microenvironment that supports chronic inflammation and tissue remodelling[23, 26].

Taken together, the development of endometriosis can be viewed as a multistep process involving dissemination of endometrial cells, impaired immune clearance, hormonal dysregulation, and activation of pro-inflammatory and pro-angiogenic pathways [4, 9, 23]. Understanding these mechanisms is essential for identifying molecular targets that may contribute to disease progression and for developing improved diagnostic and therapeutic strategies.

1.2.1 Retrograde menstruation theory

The most widely accepted explanation for the origin of endometriotic lesions is the theory of retrograde menstruation, first proposed by Sampson in the early twentieth century[22]. According to this hypothesis, menstrual debris containing viable endometrial cells flows backward through the fallopian tubes into the peritoneal cavity during menstruation. Once these cells reach the peritoneal environment, they may adhere to peritoneal surfaces, survive, and subsequently proliferate to form ectopic endometrial implants[1].

Retrograde menstruation is believed to occur in the majority of menstruating women. However, only a subset of individuals develop endometriosis, suggesting that additional biological factors determine whether ectopic endometrial cells are able to establish persistent lesions[1, 8]. In most women, immune surveillance mechanisms effectively eliminate displaced endometrial fragments. In patients with endometriosis, however, these mechanisms appear to be impaired[8].

Several cellular characteristics may contribute to the ability of endometrial cells from affected individuals to survive within the peritoneal cavity. These cells often exhibit increased adhesion to ECM components, enhanced proliferative capacity, and resistance to apoptosis[2]. Alterations in the expression of adhesion molecules such as integrins and cadherins may facilitate attachment of endometrial cells to peritoneal surfaces[2].

Additionally, ectopic endometrial cells may produce enzymes involved in ECM degradation, including matrix metalloproteinases (MMPs), which allow them to invade surrounding tissues. Increased expression of MMP-2 and MMP-9 has been reported in endometriotic lesions and is thought to contribute to their invasive behaviour[27].

Angiogenesis represents another essential step in lesion establishment. Ectopic endometrial cells and associated immune cells produce pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). These molecules stimulate endothelial cell proliferation and promote the formation of new blood vessels that supply oxygen and nutrients to developing lesions[28].

Thus, although retrograde menstruation provides a plausible mechanism for the initial dissemination of endometrial cells, the establishment of endometriotic lesions requires a combination of altered cellular properties, immune dysfunction, and pro-angiogenic signalling[1, 8, 9].

1.2.2 Coelomic metaplasia and stem cell theories

Although the theory of retrograde menstruation explains many cases of endometriosis, it does not account for all clinical observations. For example, endometriotic lesions have occasionally been reported in individuals without a functional uterus, as well as in distant anatomical locations such as the lungs or brain. These findings have led to the development of alternative hypotheses regarding the origin of ectopic endometrial tissue[1, 29].

One such hypothesis is the theory of coelomic metaplasia. According to this concept, peritoneal mesothelial cells may undergo transformation into endometrial-like cells under specific hormonal or inflammatory conditions. Both the peritoneal mesothelium and the endometrial epithelium originate from the coelomic epithelium during embryonic development, which may explain their potential for cellular plasticity[2, 30].

Another proposed mechanism involves the contribution of stem or progenitor cells. Endometrial tissue contains populations of stem-like cells capable of regenerating the endometrial lining during each menstrual cycle. These progenitor cells may enter the circulation and migrate to ectopic locations where they differentiate into endometrial-like tissue[31, 32].Evidence supporting the stem cell hypothesis includes the identification of stem cell markers such as CD146 and PDGFR-β in endometriotic lesions. Additionally, bone marrow-derived stem cells have been shown to contribute to endometrial regeneration, suggesting that circulating progenitor cells may participate in the formation of ectopic lesions[32, 33].

These alternative theories emphasize that endometriosis may arise from multiple cellular sources and that different mechanisms may contribute to lesion formation in different patients[1, 31].

1.2.3 Hormonal dysregulation

Hormonal dysregulation represents one of the central mechanisms underlying the development and progression of endometriosis. The disease is widely recognized as an estrogen-dependent condition in which altered hormonal signalling promotes the survival and proliferation of ectopic endometrial tissue. Estrogens stimulate cell proliferation, enhance inflammatory responses, and support angiogenesis within endometriotic lesions[1-3, 9].

In women with endometriosis, increased local estrogen production has been observed within ectopic lesions. One of the key mechanisms responsible for this phenomenon is the elevated expression of aromatase, the enzyme that catalyses the conversion of androgens into

estrogens. Unlike normal endometrial tissue, which exhibits limited aromatase activity, endometriotic lesions often show significantly increased expression of this enzyme, leading to enhanced local synthesis of estradiol[2, 34]. Local estrogen production is further amplified by a positive feedback loop involving PGE2. PGE2 stimulates aromatase expression in endometriotic stromal cells, thereby increasing estrogen synthesis, while estrogen in turn promotes further PGE2 production through activation of cyclooxygenase-2 (COX-2). This positive feedback mechanism contributes to sustained estrogen signalling within the lesion microenvironment[34, 35].

Another characteristic feature of endometriosis is progesterone resistance. Under normal physiological conditions, progesterone regulates endometrial differentiation and suppresses inflammatory responses during the menstrual cycle. In endometriosis, however, endometrial cells often exhibit reduced responsiveness to progesterone signalling[36]. This impaired progesterone response has been associated with decreased expression of progesterone receptor isoforms, particularly progesterone receptor B (PR-B)[36]. Progesterone resistance disrupts the normal balance between estrogen and progesterone signalling pathways. As a result, estrogen-mediated proliferative and inflammatory processes remain insufficiently controlled. This hormonal imbalance contributes to increased cellular proliferation, enhanced inflammatory signalling, and reduced apoptosis within ectopic lesions[1, 36]. Furthermore, altered expression of estrogen receptor subtypes has been reported in endometriotic tissue. Increased expression of estrogen receptor β (ERβ) relative to estrogen receptor α (ERα) has been observed in endometriotic stromal cells. ERβ signalling is thought to promote inflammatory responses and inhibit apoptosis, thereby supporting the persistence of ectopic endometrial tissue[2, 35].

Together, these hormonal alterations create a microenvironment that favours the survival, proliferation, and invasion of ectopic endometrial cells. Understanding the molecular mechanisms underlying hormonal dysregulation in endometriosis is therefore essential for identifying potential therapeutic targets and developing more effective treatment strategies[1, 9].

1.2.4 Angiogenesis and neuroangiogenesis

For endometriotic lesions to survive and expand, they must establish an adequate blood supply. Angiogenesis, defined as the formation of new blood vessels from pre-existing vasculature, is therefore a critical process in the development and maintenance of ectopic lesions[28, 37, 38].

Endometriotic tissue produces a wide range of pro-angiogenic factors that stimulate endothelial cell proliferation and migration. Among the most important of these factors is VEGF, which plays a central role in regulating vascular development. Elevated levels of VEGF have been detected in both endometriotic lesions and PF of patients with the disease[28, 37, 38]. In addition to VEGF, other angiogenic mediators such as FGF, PDGF, and angiopoietins have also been implicated in endometriosis-associated vascular remodelling[37, 38].

Hypoxia within developing lesions represents another important stimulus for angiogenesis. Hypoxic conditions activate hypoxia-inducible factor-1α (HIF-1α), a transcription factor that regulates the expression of several genes involved in angiogenic signalling, including VEGF. Activation of the HIF-1α pathway therefore contributes to the adaptation of ectopic endometrial cells to the hypoxic peritoneal environment[28, 39].

In addition to angiogenesis, neuroangiogenesis plays a significant role in the pathophysiology of endometriosis-associated pain. Neuroangiogenesis refers to the coordinated growth of nerve fibres and blood vessels within tissues. Endometriotic lesions often contain newly formed sensory and sympathetic nerve fibres capable of transmitting nociceptive signals[40, 41].

Several neurotrophic factors, including NGF and brain-derived neurotrophic factor (BDNF), have been detected in endometriotic tissue. These molecules promote nerve fibre growth and contribute to increased pain sensitivity[40, 41]. Furthermore, inflammatory mediators present within the lesion microenvironment can sensitize nociceptive neurons, amplifying pain perception[40, 41].

The combined processes of angiogenesis and neuroangiogenesis therefore contribute both to lesion survival and to the chronic pelvic pain experienced by many patients with endometriosis.

1.2.5 ECM remodelling

Remodelling of the ECM represents another essential component of endometriosis pathophysiology. The ECM provides structural support for tissues and regulates important cellular processes such as adhesion, migration, proliferation, and differentiation[4, 9]. During the development of endometriotic lesions, ectopic endometrial cells must attach to peritoneal surfaces and invade surrounding tissues. This process requires extensive remodelling of the ECM. Alterations in ECM composition facilitate the adhesion of endometrial cells and enable their migration into adjacent tissues[8, 9].

MMPs play a key role in this process. These enzymes are responsible for degrading components of the ECM, including collagen and other structural proteins[42, 43]. Increased expression of several MMPs, particularly MMP-2 and MMP-9, has been reported in endometriotic lesions[43-45]. The activity of MMPs is normally regulated by tissue inhibitors of metalloproteinases (TIMPs). In endometriosis, however, the balance between MMPs and TIMPs may be disrupted, leading to excessive matrix degradation[42, 44]. This imbalance facilitates tissue invasion and contributes to the establishment of ectopic implants.

In addition to matrix degradation, ECM remodelling influences multiple signalling pathways involved in inflammation and cellular proliferation[4, 42]. Interactions between endometrial cells and ECM components can activate intracellular signalling cascades that promote cell survival and migration[4, 9]. Furthermore, integrins and other cell adhesion molecules play important roles in mediating interactions between endometrial cells and the ECM. Altered expression of integrins has been observed in endometriotic tissue and may contribute to the enhanced adhesive properties of ectopic endometrial cells[8].

Through these mechanisms, ECM remodelling supports the invasive potential of endometrial cells and facilitates the formation of stable lesions within the peritoneal cavity.

1.2.6 Integrated view of disease mechanisms

Taken together, the mechanisms described above illustrate the complex and multifactorial nature of endometriosis. The disease arises from a combination of hormonal dysregulation, immune dysfunction, inflammatory signalling, angiogenesis, and EMC remodelling[2, 4, 9].

Importantly, these biological mechanisms do not operate independently. Instead, they form an interconnected network of signalling pathways that collectively drive disease progression[2, 4]. For example, estrogen signalling can stimulate the production of inflammatory cytokines, which in turn promote angiogenesis and matrix remodelling[2, 9]. Similarly, immune cell activation can enhance the release of growth factors that support lesion development[4]. These interconnected mechanisms are schematically summarized in Figure 1.

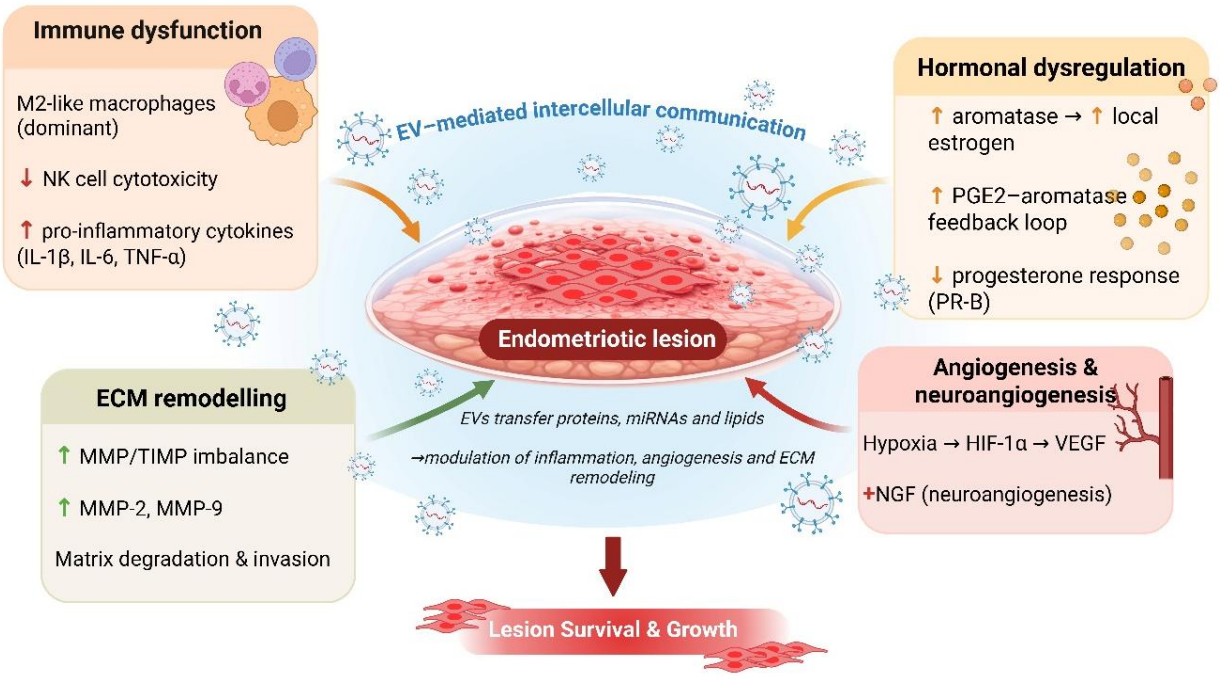

Figure 1. Integrated overview of mechanisms underlying endometriosis development and progression. Endometriotic lesion formation and persistence are driven by a complex interplay of immune dysfunction, hormonal dysregulation, angiogenesis and neuroangiogenesis, and ECM remodelling. Impaired immune surveillance, characterized by reduced natural killer (NK) cell cytotoxicity and increased pro-inflammatory cytokine production, supports lesion survival. Hormonal alterations, including increased local estrogen synthesis via aromatase and progesterone resistance, further promote proliferation and inflammatory signalling. Hypoxia-induced activation of HIF-1α and subsequent VEGF expression contribute to angiogenesis, while neurotrophic factors such as NGF support neuroangiogenesis. Concurrently, ECM remodelling, driven by an imbalance between MMPs and TIMPs, facilitates tissue invasion and lesion establishment. EVs mediate intercellular communication by transferring proteins, microRNAs (miRNAs) and lipids between cells, thereby coordinating inflammation, angiogenesis and ECM remodelling within the peritoneal microenvironment, ultimately promoting lesion survival and growth.

Within the peritoneal cavity, endometrial cells, immune cells, stromal cells, and endothelial cells engage in continuous molecular communication[2]. This communication is mediated by soluble signalling molecules such as cytokines and growth factors, as well as by EVs capable of transferring proteins, nucleic acids, and lipids between cells[17].

In recent years EVs have emerged as important mediators of intercellular signalling in many physiological and pathological processes[17]. Because EV cargo reflects the molecular state of the cells that release them, these vesicles represent a valuable source of information about ongoing biological processes within tissues[17].

Investigating the molecular composition of EVs may therefore provide new insights into the mechanisms underlying endometriosis and may contribute to the identification of novel diagnostic biomarkers[17].

1.3. Immune dysregulation in endometriosis

Immune dysregulation is considered one of the key mechanisms contributing to the development and persistence of endometriosis[2, 4, 9, 23]. Under physiological conditions, immune surveillance mechanisms present in the peritoneal cavity are capable of recognizing and eliminating misplaced endometrial cells that may enter the peritoneal space during retrograde menstruation[4, 23]. In women with endometriosis, however, this immune clearance appears to be impaired, allowing ectopic endometrial cells to survive, adhere to peritoneal surfaces, and eventually form persistent lesions[2, 4].

The peritoneal environment in patients with endometriosis is characterized by a chronic inflammatory state involving both innate and adaptive immune responses[2, 23]. Elevated concentrations of inflammatory cytokines, chemokines, and growth factors have been detected in PF, including IL-1β, IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 (MCP-1), and VEGF[2 23]. These mediators contribute to immune cell recruitment, angiogenesis, and maintenance of the inflammatory microenvironment that supports lesion development.

In addition to soluble inflammatory mediators, multiple immune cell populations participate in the pathogenesis of endometriosis. Alterations in the activity and phenotype of macrophages, NK cells, T lymphocytes (T cells), and dendritic cells have been reported in both peripheral blood and the peritoneal cavity of affected individuals[2, 23]. Importantly, immune cells within the peritoneal cavity interact closely with ectopic endometrial cells and stromal cells through a complex network of signalling pathways. Cytokines, growth factors, and EVs released by these cells participate in bidirectional communication that modulates immune responses, angiogenesis, and tissue remodelling[17]. As a result, immune dysregulation in endometriosis involves not only altered immune cell activity but also changes in intercellular communication within the peritoneal microenvironment.

1.3.1 Macrophage activation and polarization

Macrophages represent the most abundant immune cell population within the peritoneal cavity and play a central role in the pathophysiology of endometriosis[23, 46, 47]. In patients with the disease, both the number and activity of peritoneal macrophages are increased. These cells accumulate in the vicinity of endometriotic lesions and contribute to the inflammatory microenvironment through the production of cytokines, growth factors, and proteolytic enzymes[23, 46].

Macrophages exhibit remarkable functional plasticity and can adopt different activation states depending on environmental signals. Two broadly defined phenotypes are commonly described: classically activated (M1) macrophages and alternatively activated (M2) macrophages[47, 48]. M1 macrophages are typically associated with pro-inflammatory responses and produce cytokines such as IL-1β, IL-6, and TNF-α[47, 48]. In contrast, M2 macrophages are involved in tissue repair, angiogenesis, and immune regulation[48, 49].

In endometriosis, macrophage populations often display features resembling the M2 phenotype[49-51]. These alternatively activated macrophages produce factors such as IL-10, transforming growth factor-β (TGF-β), and VEGF, which promote angiogenesis, ECM remodelling, and immune tolerance within the lesion microenvironment[49-51]. As a result, macrophages contribute not only to inflammation but also to processes that support lesion survival and growth.

Macrophages may also facilitate the invasion of ectopic endometrial cells through the production of matrix metalloproteinases, including MMP-2 and MMP-9, which degrade ECM components and promote tissue remodelling[52, 53]. Furthermore, macrophages can release EVs carrying signalling molecules that influence the behaviour of surrounding cells.

1.3.2 NK cell dysfunction

NK cells are important components of the innate immune system and play a critical role in eliminating abnormal or transformed cells[54]. Under normal conditions, NK cells recognize and destroy target cells through cytotoxic mechanisms involving perforin and granzymes[54]. Several studies have demonstrated that NK cell activity is reduced in women with endometriosis [23, 55]. Both the number and cytotoxic function of NK cells may be altered, particularly within the peritoneal cavity[23, 55]. Reduced NK cell cytotoxicity impairs the immune system's ability to eliminate ectopic endometrial cells, thereby facilitating lesion establishment[23, 55].

Changes in NK cell receptor expression may contribute to this reduced cytotoxic activity. Decreased expression of activating receptors such as NKG2D and NKp46 has been reported in NK cells from patients with endometriosis, while increased expression of inhibitory receptors including certain killer immunoglobulin-like receptors (KIRs) may further suppress NK cell activation[23, 56].

In addition, the inflammatory microenvironment of the peritoneal cavity may directly influence NK cell function. Cytokines such as TGF-β and IL-10 can suppress NK cell cytotoxic activity and promote immune tolerance toward ectopic endometrial tissue[56, 57].

1.3.3 T cell imbalance

Adaptive immune responses also contribute to the pathophysiology of endometriosis[23]. Alterations in T cell populations have been observed in both peripheral blood and PF of affected patients[23, 58]. In particular, imbalances between different T helper (Th) cell subsets may influence the inflammatory environment within the peritoneal cavity.

Several studies suggest that the Th1/Th2 balance may be altered in endometriosis[23, 58, 59]. Increased production of Th2-associated cytokines such as IL-4 and IL-10 has been reported, which may contribute to immune tolerance toward ectopic tissue[58, 59]. Additionally, increased numbers of regulatory T cells (Tregs) have been detected in endometriotic lesions and PF[57, 59]. Tregs play an important role in maintaining immune tolerance and preventing excessive immune responses. However, increased Treg activity may suppress immune surveillance mechanisms that would normally eliminate ectopic endometrial cells[57]. As a result, the expansion of Treg populations may contribute to the persistence of endometriotic lesions.

Another T cell subset implicated in endometriosis is the Th17 population. Th17 cells produce pro-inflammatory cytokines such as IL-17, which promote inflammatory responses and recruit neutrophils to sites of inflammation[60]. Increased levels of IL-17 have been detected in PF of patients with endometriosis, suggesting that Th17-mediated inflammation may also contribute to disease progression[60].

1.3.4 Cytokine networks and inflammatory mediators

Cytokines and chemokines represent key regulators of immune signalling in endometriosis and play an essential role in shaping the inflammatory microenvironment of the peritoneal cavity[23, 61]. Elevated concentrations of numerous pro-inflammatory mediators have been detected in PF as well as in the systemic circulation of patients with the disease[23, 61, 62]. Among the most frequently reported cytokines are IL-1β, IL-6, IL-8, TNF-α, and MCP-1[23, 61, 62]. These molecules act as signalling factors that coordinate immune cell recruitment, activation, and communication within the peritoneal environment.

Cytokine networks in endometriosis influence several biological processes involved in disease development. Pro-inflammatory mediators can stimulate proliferation of endometrial stromal cells and enhance the survival of ectopic tissue[62]. For example, IL-6 and TNF-α are known to activate intracellular signalling pathways associated with cell proliferation and resistance to apoptosis[11]. At the same time, chemokines such as MCP-1 promote the recruitment of monocytes and macrophages to the peritoneal cavity, further amplifying inflammatory responses[11].

Inflammatory cytokines also play an important role in angiogenesis and tissue remodelling. Several cytokines can stimulate the production of pro-angiogenic factors such as VEGF, which promotes the formation of new blood vessels necessary for lesion survival[37].In addition, inflammatory signalling can increase the expression of MMPs, including MMP-2 and MMP-9, which participate in ECM degradation and facilitate the invasion of ectopic endometrial cells into surrounding tissues[37].

Beyond their role in inflammation and tissue remodelling, cytokines may also influence neuronal signalling pathways associated with endometriosis-related pain[63]. Certain inflammatory mediators, including IL-1β, IL-6, and TNF-α, are capable of sensitizing nociceptive nerve fibres and enhancing their responsiveness to mechanical and chemical stimuli[63]. This process, often referred to as peripheral sensitization, contributes to the chronic pelvic pain experienced by many patients with endometriosis.

Altogether, cytokine networks form a complex signalling system that integrates immune activation, angiogenesis, ECM remodelling, and neuroinflammatory processes. Through these interconnected mechanisms, inflammatory mediators contribute both to the establishment of endometriotic lesions and to the clinical manifestations of the disease.

1.3.5 Interaction between immune cells and the peritoneal microenvironment

The peritoneal cavity represents a complex and dynamic microenvironment in which immune cells, endometrial cells, stromal cells, and endothelial cells interact through multiple signalling pathways. Within this environment, a wide range of soluble mediators and cellular signals regulate inflammatory responses, angiogenesis, and tissue remodelling. These interactions are essential for understanding the biological mechanisms that support the establishment and persistence of endometriotic lesions[4, 9, 64, 65].

Ectopic endometrial cells are not passive components of this microenvironment but actively participate in shaping immune responses. These cells secrete numerous cytokines,

chemokines, and growth factors, including IL-6, IL-8, TNF-α, and VEGF. Through the release of these mediators, endometrial cells can recruit immune cells to the peritoneal cavity and influence their activation state[11, 23, 64].

In turn, immune cells present within the peritoneal cavity, particularly macrophages, dendritic cells, and T cells, produce mediators that influence the proliferation, survival, and migration of endometrial cells. Macrophage-derived cytokines and growth factors may stimulate angiogenesis and ECM remodelling, while T cell-derived cytokines can modulate inflammatory signalling pathways within ectopic lesions[9, 23, 64]. This bidirectional communication between endometrial cells and immune cells creates a feedback loop that sustains chronic inflammation and promotes lesion development[4, 9, 64]. These interactions within the peritoneal microenvironment are schematically summarized in Figure 2.

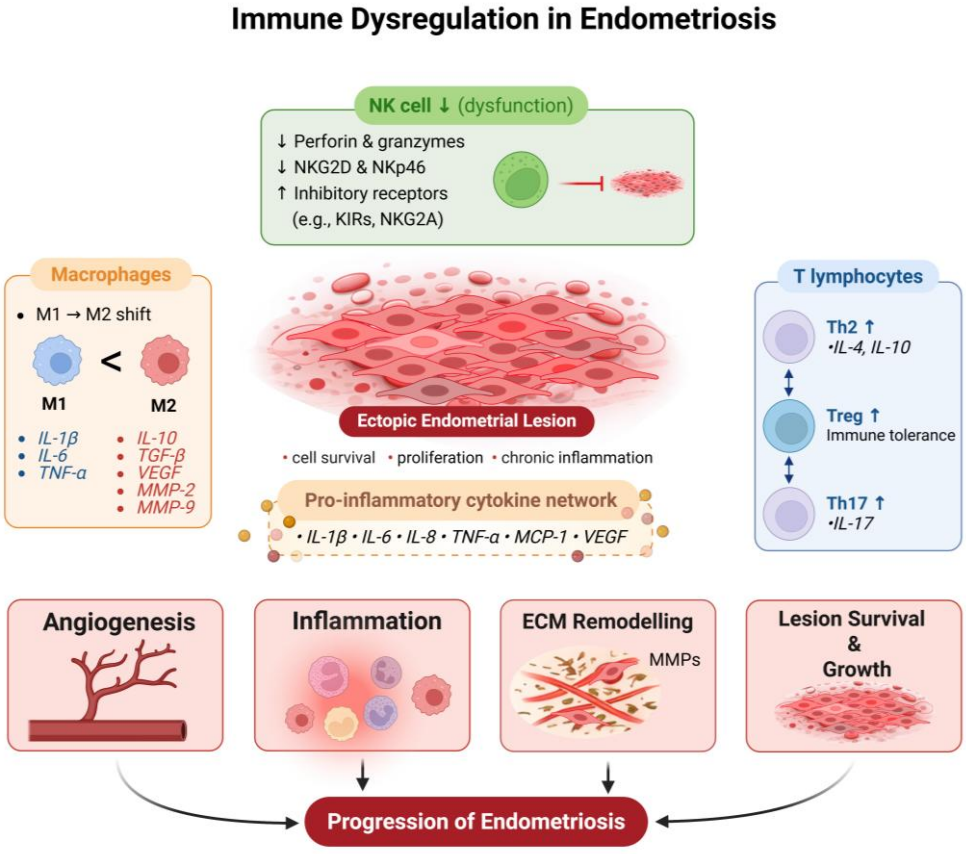

Figure 2. Interaction between immune cells and the peritoneal microenvironment in endometriosis. The peritoneal cavity constitutes a dynamic microenvironment in which ectopic endometrial cells interact with immune cells, including macrophages, NK cells and T cells. Macrophages exhibit a shift towards an M2-like phenotype, associated with increased production of anti-inflammatory and pro-angiogenic mediators such as IL-10, TGF-β, VEGF and MMPs, while pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) contribute to a persistent inflammatory state. NK cells display reduced cytotoxic activity, characterized by decreased expression of perforin, granzymes and activating receptors, alongside increased inhibitory signalling. Altered T cell responses, including increased Th2, Treg and Th17 activity, promote immune tolerance and chronic inflammation. These interactions are mediated by a pro-inflammatory cytokine network that supports angiogenesis, ECM remodelling and lesion survival, ultimately contributing to disease progression.

In recent years, increasing attention has been directed toward the mechanisms by which molecular signals are transferred between cells within the lesion microenvironment. While soluble mediators such as cytokines and chemokines represent an important form of intercellular communication, growing evidence indicates that EVs also play a significant role in mediating cell-to-cell signalling[17, 66, 67].

EVs released by endometrial cells, immune cells, and other cell types present in the peritoneal cavity may carry proteins, lipids, messenger RNA (mRNA), and miRNAs capable of influencing the behaviour of recipient cells. Through the transfer of this molecular cargo, EVs can modulate immune responses, regulate inflammatory signalling pathways, and influence processes such as angiogenesis and tissue remodelling[17, 66-68]. As a result, EV-mediated communication may contribute to the establishment and maintenance of the inflammatory microenvironment characteristic of endometriosis.

Understanding the interactions between immune cells and extracellular vesicle signalling pathways may therefore provide important insights into the molecular mechanisms underlying disease progression. Moreover, molecules associated with EV-mediated communication may represent promising candidates for the development of novel diagnostic biomarkers and therapeutic strategies[17, 66, 67].

1.4. Diagnostic challenges and the need for biomarkers in endometriosis

Despite significant advances in understanding the biological mechanisms underlying endometriosis, the diagnosis of the disease remains a major clinical challenge. One of the most problematic aspects of endometriosis management is the substantial delay between the onset of symptoms and the establishment of a definitive diagnosis. In many patients this delay may extend for several years, during which the disease may progress and symptoms can become increasingly severe[4, 69].

Several factors contribute to this prolonged diagnostic interval. Many symptoms are non-specific and may overlap with other gynaecological or gastrointestinal disorders. Consequently, they are frequently attributed to more common conditions or considered a normal component of the menstrual cycle. This diagnostic uncertainty often results in repeated medical consultations before appropriate investigations are initiated[4, 69]. As mentioned previously, another major factor contributing to diagnostic delay is the lack of reliable non-invasive diagnostic tools[69-71].

Another challenge arises from the biological and morphological heterogeneity of endometriotic lesions. Lesions may differ in terms of size, anatomical location, depth of infiltration, and molecular characteristics. Some lesions appear as superficial peritoneal implants, whereas others form ovarian cysts known as endometriomas or deeply infiltrating nodules that penetrate surrounding tissues. This heterogeneity complicates the development of universal diagnostic criteria and further contributes to difficulties in disease detection[4, 69]. Given these limitations, considerable research efforts have focused on identifying molecular biomarkers that could enable earlier and less invasive diagnosis of endometriosis. Ideally, such biomarkers should be detectable in easily accessible biological fluids, exhibit high sensitivity and specificity, and reflect biological processes occurring within endometriotic lesions[70, 71].

Over the past decades numerous candidate biomarkers have been investigated, including inflammatory cytokines, growth factors, hormones, and proteins involved in ECM remodelling[11, 23]. Among these candidates, the glycoprotein cancer antigen 125 (CA-125) has attracted considerable attention. Elevated CA-125 concentrations have been reported in some patients with endometriosis, particularly in advanced stages of the disease. However, the diagnostic performance of CA-125 remains limited, as increased levels can also occur in other gynaecological disorders and inflammatory conditions. Consequently, CA-125 lacks sufficient sensitivity and specificity to serve as a reliable standalone diagnostic marker[70, 72]. In addition to individual biomarkers, several studies have explored the potential of multi-marker panels combining different biological molecules. Although such approaches may improve diagnostic accuracy, many proposed biomarker panels have not yet demonstrated sufficient reproducibility across independent studies[70, 73].

Recent advances in molecular technologies have opened new opportunities for biomarker discovery. High-throughput approaches such as transcriptomics, proteomics, and metabolomics enable comprehensive analysis of molecular profiles associated with disease processes. These technologies allow the identification of complex molecular signatures rather than relying on individual biomarkers[74]. Particular attention has recently been directed toward circulating molecular signals present in biological fluids. Blood, PF, urine, and other body fluids contain a wide range of molecules released by cells throughout the body. Analysis of these molecules may provide valuable information about pathological processes occurring within specific tissues[74, 75].

Among the various carriers of circulating molecular information, EVs have emerged as particularly promising candidates for biomarker discovery. EVs are membrane-bound particles released by virtually all cell types and are present in numerous biological fluids[17, 66, 67]. Because EVs contain proteins, lipids, mRNA, and miRNAs derived from their cells of origin, their molecular composition may reflect physiological and pathological processes occurring within tissues[66-68]. In addition to serving as carriers of molecular information, EVs participate in intercellular communication by transferring bioactive molecules between cells. Through this mechanism EVs may influence processes such as inflammation, angiogenesis, immune regulation, and ECM remodelling, all of which are known to play key roles in the development and progression of endometriosis[17, 66-68]. Because EVs are present in both local biological compartments, such as PF, and systemic circulation, they may provide insight into molecular processes occurring within the peritoneal microenvironment as well as in the whole organism[17, 67]. Investigating the molecular composition of EV populations derived from different biological fluids may therefore facilitate the identification of disease-associated molecular signatures.

For these reasons, EVs have become an increasingly important focus of research in the field of endometriosis. Understanding their biological functions and molecular composition may contribute not only to improved knowledge of disease mechanisms but also to the development of novel minimally invasive diagnostic strategies[17, 66-68].

1.5. EVs: biology, classification and molecular cargo

EVs are membrane-bound particles released by cells into the extracellular environment and are now widely recognized as important mediators of intercellular communication. Nearly all cell types are capable of producing EVs under both physiological and pathological conditions. These vesicles are surrounded by a lipid bilayer membrane and contain a diverse range of bioactive molecules, including proteins, lipids, mRNA, miRNA, and fragments of genomic or mitochondrial DNA. Through the transfer of these molecules between cells, EVs can influence numerous biological processes such as immune responses, inflammation, angiogenesis, and tissue remodelling[17, 19, 67, 76, 77].

For many years EVs were considered cellular debris or byproducts of membrane turnover. However, advances in molecular biology, high-resolution microscopy, and high-throughput analytical technologies have fundamentally changed this perception. It is now well established that EVs represent an active mechanism of cell-cell communication through which

cells exchange molecular information and modulate the behaviour of neighbouring or distant cells[17, 19, 67, 77].

One of the defining characteristics of EVs is that their molecular cargo reflects the physiological and pathological state of their cells of origin. Cellular stress, metabolic alterations, inflammatory signalling, or disease-associated molecular pathways may influence both the quantity and the molecular composition of EVs released by cells. Consequently, EV populations present in biological fluids can provide valuable insight into biological processes occurring within tissues[17, 19, 67, 77].

EVs are widely distributed in a variety of biological fluids, including blood, urine, saliva, cerebrospinal fluid, breast milk, and PF. Their presence in both local and systemic compartments allows them to participate in complex communication networks throughout the body. Importantly, the lipid bilayer membrane surrounding EVs protects their molecular cargo from enzymatic degradation, allowing proteins and nucleic acids associated with EVs to remain stable even in complex biological fluids[17, 19, 67, 77].

1.5.1 Classification of EVs

EVs are commonly classified into several subtypes based on their biogenesis and approximate size. The most frequently described categories include exosomes, microvesicles, and apoptotic bodies. The classification and main biogenesis pathways of EVs are schematically illustrated in Figure 3. Although this classification provides a useful conceptual framework, it should be noted that EV populations are highly heterogeneous and often overlap in size, density, and molecular composition[19, 67, 77].

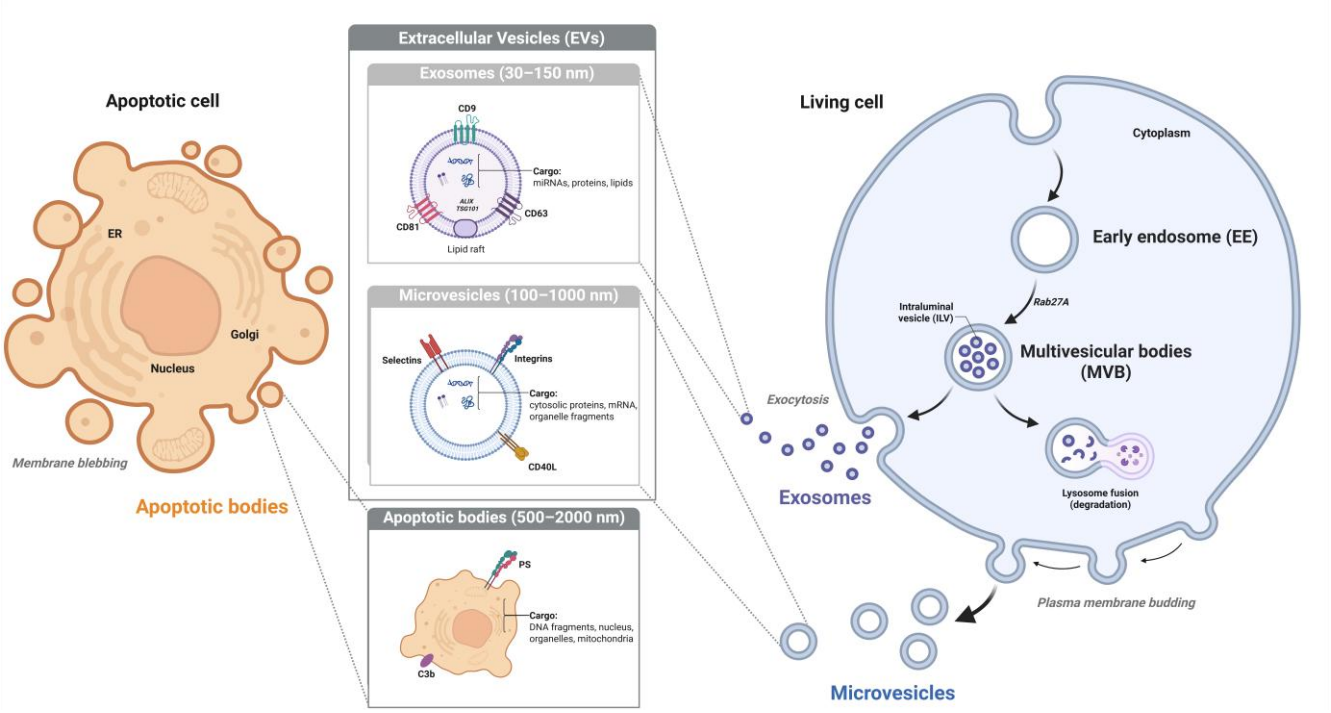

Figure 3. Classification and biogenesis of EVs. EVs are commonly classified into three main subtypes: exosomes (30-100 nm), microvesicles (100-1000 nm), and apoptotic bodies (500-2000 nm), which differ in their size and mechanisms of formation. Exosomes originate from the endosomal pathway through the formation of multivesicular bodies (MVBs) and are released upon fusion with the plasma membrane. Microvesicles are generated by direct outward budding of the plasma membrane, whereas apoptotic bodies are released during programmed cell death. Despite this classification, EV populations are highly heterogeneous and exhibit overlapping physical and molecular characteristic

Exosomes are generally considered a subtype of EVs generated through the endosomal pathway. Their formation begins with the internalization of plasma membrane components through endocytosis, resulting in the formation of early endosomes. As these endosomes mature into late endosomes, inward budding of the endosomal membrane leads to the formation of intraluminal vesicles within structures known as MVBs. When MVBs fuse with the plasma membrane, these intraluminal vesicles are released into the extracellular space and are referred to as exosomes[19, 67, 77].

The formation of intraluminal vesicles within MVBs is regulated by several molecular mechanisms. One of the best-characterized pathways involves the endosomal sorting complex required for transport (ESCRT), a group of protein complexes responsible for cargo sorting and membrane remodelling. Key ESCRT-associated proteins frequently detected in EV preparations include tumour susceptibility gene 101 (TSG101) and apoptosis-linked gene 2-interacting protein X (ALIX)[19, 67, 77]. In addition to ESCRT-dependent mechanisms, ESCRT-independent pathways involving lipids such as ceramides have also been implicated in exosome biogenesis[19, 77].

Microvesicles, sometimes referred to as ectosomes, are generated through direct outward budding of the plasma membrane. This process involves coordinated rearrangement of cytoskeletal elements, changes in membrane lipid composition, and redistribution of phospholipids such as phosphatidylserine (PS). Microvesicles are generally larger than exosomes, although substantial overlap exists between these vesicle populations[19, 77].

Apoptotic bodies represent another category of EVs and are produced during programmed cell death. These vesicles tend to be larger than other EV types and may contain fragments of cellular organelles, chromatin, or nuclear material. Although apoptotic bodies arise from a distinct biological process, they also contribute to the heterogeneous population of EVs present in biological fluids[67, 77].

Because distinguishing between different EV subtypes remains technically challenging, many studies prefer to use the broader term "EVs" to describe all membrane-bound particles released by cells. This terminology reflects the current consensus within the EV research community and is consistent with recommendations proposed by the International Society for Extracellular Vesicles (ISEV)[76]. Using the general term EVs avoids assumptions regarding the precise origin or biogenesis of vesicles when such information cannot be definitively established.

1.5.2 Biogenesis of EVs

The formation of EVs is a tightly regulated cellular process involving multiple molecular pathways responsible for membrane remodelling, cargo selection, and vesicle release[66, 67, 76, 77]. The biogenesis of exosomes is primarily associated with the endosomal system and involves the formation of multivesicular bodies (MVBs)[19, 66, 77]. During this process, specific proteins, lipids, and nucleic acids are selectively incorporated into intraluminal vesicles that later become exosomes upon release into the extracellular space[19, 77].

One of the best-characterized mechanisms involved in exosome formation is the endosomal sorting complex required for transport (ESCRT) machinery[19, 78]. The ESCRT system consists of several protein complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) that coordinate cargo sorting and membrane budding within endosomes[19, 78]. These complexes facilitate inward budding of the endosomal membrane and contribute to the selective incorporation of specific proteins into developing vesicles[19, 78]. Proteins frequently associated with the ESCRT pathway and commonly detected in EV preparations include

tumour susceptibility gene 101 (TSG101) and apoptosis-linked gene 2-interacting protein X (ALIX)[76, 78].

In addition to ESCRT-dependent mechanisms, ESCRT-independent pathways have also been described[77]. These mechanisms involve specific lipids and membrane proteins that contribute to membrane curvature and vesicle formation[79]. For example, sphingolipid metabolism leading to the generation of ceramide has been shown to promote exosome formation by facilitating membrane invagination within endosomes[79]. Tetraspanin proteins such as CD9, CD63, and CD81 may also participate in organizing membrane microdomains that influence vesicle formation and cargo selection[77].

Microvesicle formation occurs through a different mechanism involving direct outward budding of the plasma membrane[67, 80]. This process requires coordinated rearrangement of cytoskeletal structures and alterations in membrane lipid asymmetry[67]. Increased intracellular calcium levels can activate enzymes such as scramblases and calpain, which contribute to the redistribution of phospholipids such as phosphatidylserine across the plasma membrane[80]. These changes promote membrane curvature and ultimately lead to the release of microvesicles into the extracellular environment[67, 80].

Importantly, the rate and composition of EV production can be influenced by various physiological and pathological stimuli[17, 77]. Cellular stress, hypoxia, inflammatory signalling, and exposure to growth factors may alter the activity of vesicle biogenesis pathways and affect the molecular composition of EV cargo[17, 77]. As a result, EV populations released under different biological conditions may exhibit distinct molecular profiles that reflect the physiological state of the producing cells[77].

1.5.3 Molecular cargo of EVs

One of the most distinctive features of EVs is the diversity of molecules contained within their cargo. EVs carry a complex mixture of proteins, lipids, and nucleic acids derived from their cells of origin. This molecular cargo can influence the behaviour of recipient cells following EV uptake and therefore represents an important mechanism of intercellular communication[19, 67, 76, 77].

Proteins constitute a major component of EV cargo. These proteins include structural membrane proteins, enzymes involved in metabolic processes, signalling molecules, and proteins associated with cell adhesion and immune regulation[19, 76, 77]. Several protein families are commonly enriched in EV populations and are frequently used as markers of EVs. These

include tetraspanins such as CD9, CD63, and CD81, as well as cytosolic proteins involved in vesicle trafficking, including TSG101 and ALIX[76, 77, 81]. Additional proteins often detected in EVs include heat shock proteins (HSPs), including HSP70 and HSP90, cytoskeletal proteins, integrins, and proteins involved in immune signalling pathways[19, 76, 81].

EVs also contain various nucleic acids, including mRNA, miRNA, and other non-coding RNAs[19, 67, 78]. These molecules can regulate gene expression in recipient cells following EV internalization. For example, miRNAs delivered by EVs, including exosomes, may suppress translation of specific target mRNAs and thereby influence cellular signalling pathways involved in proliferation, inflammation, or differentiation[67, 82].

Lipids represent another key component of EV cargo. The lipid composition of EV membranes contributes to vesicle stability and influences interactions with recipient cells. EV membranes are often enriched in cholesterol, sphingomyelin, and ceramides, which help maintain membrane rigidity and structural integrity[83]. In addition to their structural roles, certain lipids can participate directly in signalling pathways associated with inflammation and immune regulation[83].

The selective incorporation of specific molecules into EV cargo indicates that vesicle formation is not a random process. Instead, cells possess mechanisms that allow selective sorting of proteins, RNAs, and lipids into EVs[67, 78]. These sorting processes enable cells to package and transmit specific molecular signals to neighbouring or distant cells within their microenvironment.

1.5.4 EVs as mediators of intercellular communication

EVs play a significant role in intercellular communication by transferring their molecular cargo between cells[19, 67, 76, 77]. After being released into the extracellular environment, EVs can interact with recipient cells through several mechanisms, including direct fusion with the plasma membrane, receptor-mediated binding, or internalization through endocytic pathways such as clathrin-mediated endocytosis, macropinocytosis, or phagocytosis[66, 67].

Following uptake by recipient cells, EV cargo can influence a variety of cellular processes. Proteins delivered by EVs may activate intracellular signalling pathways, whereas transferred RNA molecules can regulate gene expression at the post-transcriptional leve[67, 82]. Through these mechanisms, EVs are capable of modulating the behaviour of recipient cells and coordinating biological responses within tissues.

EV-mediated communication plays essential roles in many physiological processes, including immune regulation, tissue repair, angiogenesis, and development[19, 76, 77]. At the same time, this communication system can also contribute to pathological processes. In several diseases, including cancer, neurodegenerative disorders, and inflammatory conditions, EVs have been shown to facilitate the spread of disease-associated molecular signals between cells[66, 67].

In the context of endometriosis, EVs may participate in communication between endometrial cells, immune cells, endothelial cells, and stromal cells within the peritoneal cavity. Through the transfer of proteins and regulatory RNAs, EVs may influence inflammatory signalling, angiogenesis, immune responses, and ECM remodelling[18, 68]. These processes are known to play central roles in the development and progression of endometriotic lesions. Beyond their biological functions, EVs have attracted considerable interest as potential diagnostic biomarkers. Because EVs are present in numerous biological fluids and carry molecular cargo derived from their cells of origin, analysis of EV-associated molecules may provide valuable information about pathological processes occurring within tissues[19, 67]. These properties make EVs promising targets for molecular studies aimed at identifying disease-associated signatures in complex biological samples.

1.6. EVs in reproductive biology and endometriosis

EVs have emerged as important mediators of intercellular communication within the reproductive system[19, 67, 76, 77]. Increasing evidence indicates that EVs participate in numerous physiological processes occurring in the female reproductive tract, including follicular development, embryo implantation, immune regulation, and tissue remodelling[84]. Because these vesicles can transfer proteins, lipids, and nucleic acids between cells, they play a crucial role in coordinating cellular interactions within reproductive tissues[67, 77].

In the female reproductive system, EVs have been detected in multiple biological fluids, including follicular fluid, uterine fluid, cervical mucus, and PF[84, 85]. These vesicles are released by various cell types such as endometrial epithelial cells, stromal cells, immune cells, and endothelial cells[86]. Through EV-mediated communication these cell populations exchange molecular signals that regulate local physiological processes and maintain tissue homeostasis[84, 86].

Within the endometrium, EVs participate in the regulation of cellular proliferation, differentiation, and immune responses throughout the menstrual cycle[77, 86]. The endometrium

undergoes dynamic cyclic remodelling under the influence of hormonal signals, and EV-mediated communication contributes to the coordination of these changes[86]. Vesicles released by endometrial cells may contain signalling molecules that influence neighbouring epithelial, stromal, and immune cells, thereby supporting tissue regeneration and functional adaptation during the menstrual cycle[77, 86].

EVs also play a significant role in processes associated with embryo implantation and early pregnancy[84, 85, 87]. Successful implantation requires precise communication between the embryo and the maternal endometrium, including modulation of immune responses and coordinated tissue remodelling. EVs released by trophoblast cells as well as by maternal endometrial cells can transfer regulatory molecules such as miRNAs and signalling proteins that influence gene expression and cellular behaviour within the implantation site[87, 88].

Given their role in reproductive physiology, it is not surprising that EVs have also been implicated in pathological conditions affecting the reproductive system. In endometriosis, EV-mediated communication may contribute to the complex interactions between ectopic endometrial cells, immune cells, stromal cells, and endothelial cells present within the peritoneal cavity[68, 89].

Endometriotic lesions develop within a dynamic microenvironment characterized by chronic inflammation, angiogenesis, and extensive ECM remodelling[68, 89]. Cells within this microenvironment continuously exchange molecular signals that regulate cellular proliferation, migration, and survival. EVs represent one of the mechanisms through which these signals can be transferred between cells[68]. EVs released by ectopic endometrial cells may influence surrounding immune cells and contribute to the establishment of a pro-inflammatory environment that favours lesion persistence[68, 89]. Conversely, EVs derived from immune cells may affect the behaviour of endometrial cells by modulating their proliferative activity, migratory potential, and invasive capacity[68, 89].

In addition to immune modulation, EVs may participate in angiogenesis associated with endometriotic lesion growth. The formation of new blood vessels is essential for lesion survival because it provides oxygen and nutrients necessary for cellular proliferation. EVs carrying pro-angiogenic molecules such as VEGF or angiogenesis-related miRNAs may stimulate endothelial cell activation and promote vascular development within the lesion microenvironment[68, 89, 90].

EVs may also contribute to ECM remodelling by transporting enzymes and regulatory molecules involved in matrix degradation and tissue invasion. Proteases such as MMPs or

signalling molecules regulating their activity may facilitate the attachment of ectopic endometrial cells to peritoneal surfaces and support the establishment of stable lesions within surrounding tissues[68, 89].

Recent studies have also highlighted the role of EV-associated miRNAs in regulating gene expression in recipient cells[68, 90]. miRNAs represent small non-coding RNA molecules that modulate gene expression at the post-transcriptional level. EV-mediated transfer of miRNAs between cells may influence signalling pathways associated with inflammation, angiogenesis, cell proliferation, and immune regulation[68, 90]. Alterations in the molecular cargo of EVs released by endometrial or immune cells may therefore contribute to pathological processes underlying endometriosis[68, 89]. Differences in EV composition between healthy individuals and patients with endometriosis may reflect changes in cellular signalling associated with disease development[68].

Because EVs are present in biological fluids such as blood and PF, analysis of their molecular cargo provides a valuable opportunity to investigate disease-associated signalling pathways[68, 91]. Studying EV populations derived from different biological compartments may therefore provide insight into both local and systemic mechanisms involved in endometriosis[91].

PF represents a particularly important biological environment because it directly reflects the local microenvironment surrounding endometriotic lesions[68, 89]. EVs present in PF may carry molecular signatures derived from cells located within the peritoneal cavity, including ectopic endometrial cells and immune cells participating in inflammatory responses[68]. At the same time, EVs circulating in the bloodstream may reflect systemic alterations associated with the disease[91]. Comparative analysis of EV populations derived from PF and plasma may therefore provide complementary information about both local and systemic aspects of endometriosis[91]. Understanding how EV-mediated communication contributes to disease progression may facilitate the identification of novel biomarkers and therapeutic targets for endometriosis.

The growing interest in EVs as mediators of disease processes has also led to increasing efforts aimed at characterizing their molecular composition using advanced analytical techniques. In particular, high-throughput proteomic approaches have become powerful tools for investigating the protein cargo of EV populations[92]. Such technologies allow the identification of complex molecular signatures that may be associated with specific pathological conditions.

1.7. EVs as liquid biopsy biomarkers

The concept of liquid biopsy has emerged as a promising strategy for the detection and monitoring of various diseases[93, 94]. Unlike traditional tissue biopsy, which requires invasive sampling of affected tissues, liquid biopsy relies on the analysis of molecular markers present in more or less easily accessible biological fluids such as blood, urine, or cerebrospinal fluid[93, 94]. These fluids contain a wide range of circulating molecules released from cells throughout the body, including nucleic acids, proteins, metabolites, and EVs[17, 94].

Liquid biopsy approaches have attracted considerable attention because they offer several advantages over conventional diagnostic methods. Sampling of biological fluids is minimally invasive and can be usually repeated multiple times during the course of disease, enabling longitudinal monitoring of pathological processes[93]. In addition, analysis of circulating molecules may provide real-time insight into dynamic biological events occurring within tissues[94]. For these reasons, liquid biopsy technologies are increasingly explored as tools for disease diagnosis, prognosis, and treatment monitoring.

Among the various carriers of circulating molecular information, EVs have emerged as particularly promising candidates for biomarker discovery[17, 77]. EVs are released by virtually all cell types and are present in numerous biological fluids, including plasma, serum, urine, saliva, and PF[17, 77]. Because EVs carry molecular cargo derived from their cells of origin, their composition may reflect physiological or pathological processes occurring within specific tissues[17].

One of the major advantages of EVs as biomarkers is the stability of their molecular cargo. The lipid bilayer membrane surrounding EVs protects proteins and nucleic acids from enzymatic degradation, which represents a common challenge in the analysis of circulating molecules[19, 95]. Consequently, EV-associated proteins, RNAs, and other molecules can remain detectable even in complex biological fluids containing numerous proteases and nucleases.
Another important feature of EVs is their ability to enrich disease-associated molecules. Cells undergoing pathological changes frequently alter both the quantity and molecular composition of the EVs they release[17]. These vesicles may contain proteins, lipids, miRNAs, and other regulatory molecules associated with disease-related signalling pathways[17, 77]. Because EVs selectively package specific molecules into their cargo, they can serve as concentrated sources of biologically relevant information.

EVs also offer the possibility of identifying biomarkers that reflect the cellular origin of vesicles. Surface proteins present on EV membranes, including tetraspanins such as CD9,

CD63, and CD81, as well as cell-type-specific markers, may provide information about the tissues or cell populations from which the vesicles were derived[77]. Analysis of such markers may therefore help identify the cellular sources contributing to the circulating EV pool in a given disease. In addition to providing information about disease presence, EV-based biomarkers may also be useful for monitoring disease progression and treatment response. Because EVs are continuously released by cells, changes in their molecular composition may reflect dynamic alterations in cellular activity[17]. Repeated sampling of biological fluids may therefore enable longitudinal monitoring of disease-associated molecular signals.

Therefore, EVs have been investigated in recent years as potential biomarkers in a wide range of diseases. In oncology, EV-associated proteins and nucleic acids have been studied as indicators of tumour presence, tumour subtype, and response to therapy[96]. In neurological disorders, EVs derived from neurons and glial cells have been detected in blood and cerebrospinal fluid, providing insight into pathological processes occurring within the central nervous system[97]. Similarly, cardiovascular diseases have been associated with changes in circulating EV populations, including vesicles released from endothelial cells, platelets, and immune cells[98].

Within gynaecological research, EVs have increasingly been explored as potential biomarkers for conditions affecting the reproductive system[84]. Studies have shown that EV populations present in reproductive tract fluids and in systemic circulation may carry molecular signatures associated with pathological processes occurring within reproductive tissues[84, 89]. For endometriosis in particular, the identification of reliable non-invasive biomarkers remains a major research priority[1]. As discussed earlier, current diagnostic strategies rely heavily on invasive surgical procedures, and existing biomarkers lack sufficient sensitivity and specificity for routine clinical use[1]. Consequently, the identification of novel biomarker sources is essential for improving early detection of the disease.

EVs possess several characteristics that make them attractive candidates for biomarker discovery in endometriosis. First, EVs are present both in local biological fluids such as PF and in systemic compartments such as plasma[84, 89]. This distribution enables the investigation of molecular signals associated with both the local microenvironment of endometriotic lesions and systemic responses to the disease. Second, the molecular cargo of EVs may reflect the biological state of endometrial cells, immune cells, and other cell types involved in the pathophysiology of endometriosis[89]. Alterations in EV-associated proteins or regulatory RNAs may therefore provide insight into inflammatory processes, angiogenesis, immune

modulation, and ECM remodelling occurring within endometriotic lesions. Third, the analysis of EVs can be combined with high-throughput molecular technologies such as proteomics, transcriptomics, and lipidomics[17]. These approaches enable comprehensive characterization of EV cargo and facilitate the identification of complex molecular signatures associated with disease processes.

Despite these promising features, the application of EV-based biomarkers also presents several technical challenges. One important limitation is the heterogeneity of EV populations present in biological fluids[77]. Vesicles released by different cell types vary in size, molecular composition, and biological function, making it difficult to distinguish disease-associated vesicles from background populations. Another challenge involves the lack of standardized protocols for EV isolation and characterization. Differences in sample preparation methods, isolation techniques, and analytical platforms may influence the composition of EV preparations and complicate comparisons between studies[77]. To address these issues, international guidelines such as the Minimal Information for Studies of Extracellular Vesicles (MISEV2023) have been developed to promote standardization and reproducibility in EV research[76].

Recent advances in analytical technologies are gradually overcoming many of these challenges. Improved methods for EV isolation, characterization, and molecular profiling have significantly expanded the possibilities for studying EVs in clinical samples[17]. In particular, high-resolution proteomic approaches allow detailed investigation of EV-associated proteins present in biological fluids. These developments have opened new opportunities for exploring EVs as sources of disease-associated molecular signatures in endometriosis[89]. By analysing EV populations derived from different biological compartments, it becomes possible to investigate both local and systemic processes involved in disease development and progression.

1.8. Proteomic profiling of EVs

Proteomic analysis has become one of the most powerful approaches for investigating the molecular composition of EVs. Advances in mass spectrometry (MS)-based proteomics have made it possible to identify and quantify hundreds to thousands of proteins in complex biological samples, including EV preparations[99-101].

The protein composition of EVs reflects both the cellular origin of vesicles and the physiological state of the cells that produce them. During vesicle formation, specific proteins

are selectively incorporated into EV cargo through regulated sorting mechanisms involving endosomal trafficking pathways and membrane microdomains[66, 78]. As a result, EV-associated proteins may provide information about intracellular signalling pathways, metabolic activity, immune responses, and cellular adaptation to environmental stimuli[17, 100]. Proteomic profiling of EVs has therefore become an important tool for studying intercellular communication and identifying potential disease biomarkers[17, 100]. By comparing EV protein profiles between different biological conditions, researchers can identify proteins that are differentially expressed or enriched in specific disease states. These proteins may represent candidate biomarkers or provide insight into biological pathways associated with disease development[17].

MS represents the central analytical technology used in modern proteomic studies[102]. In a typical proteomic workflow, proteins present in EV samples are first extracted and enzymatically digested into peptides, most commonly using trypsin. These peptides are subsequently separated by liquid chromatography and analysed using liquid chromatography–tandem mass spectrometry (LC-MS/MS), which measures the mass-to-charge ratios of peptide ions and their fragmentation spectra[102]. The resulting spectral data are then matched to protein databases using computational algorithms, enabling identification and quantification of proteins present in the sample[102]. Several quantitative proteomic strategies have been developed to compare protein abundance between biological samples. One commonly used approach is label-free quantification[103]. Another widely used strategy involves isobaric labelling techniques such as tandem mass tags (TMT)[104].

In addition to protein identification and quantification, proteomic datasets can be integrated with bioinformatic analyses to provide functional interpretation of results[105, 106]. Computational tools enable the identification of enriched biological pathways, molecular networks, and cellular processes associated with proteins detected in EV samples. Protein-protein interaction (PPI) network analysis represents another powerful strategy for interpreting proteomic data[105].

Over the past decade, proteomic studies of EVs have significantly expanded our understanding of EV biology. Large-scale databases such as Vesiclepedia and ExoCarta have been established to curate proteins, RNAs, and lipids identified in EV populations across numerous studies[107, 108]. Despite these advances, proteomic analysis of EVs presents several technical challenges, including EV isolation from complex biological fluids and the intrinsic heterogeneity of vesicle populations[17, 76]. In the context of endometriosis, proteomic profiling

of EVs represents a promising strategy for identifying disease-associated molecular signatures[18].

1.9. Single-vesicle analysis and imaging flow cytometry (IFCM) in EV research

Although bulk molecular analyses such as proteomics provide valuable information about the overall composition of EV populations, they do not fully capture the heterogeneity that exists among individual vesicles[76]. EVs present in biological fluids originate from multiple cell types and may differ in size, molecular composition, and biological function[17, 76]. As a result, EV preparations typically contain complex mixtures of vesicles with diverse cellular origins and molecular cargo.

Traditional bulk analytical approaches measure average molecular signals derived from entire vesicle populations. While such methods are highly informative for identifying global molecular patterns, they cannot distinguish between specific EV subpopulations that may carry distinct biological information[76]. Consequently, increasing attention has been directed toward analytical techniques capable of characterizing individual vesicles. Single-vesicle analysis enables investigation of EV heterogeneity at a much higher level of resolution[76, 109]. By analysing vesicles individually rather than as bulk populations, researchers can identify specific EV subsets and examine differences in surface marker expression, size distribution, and molecular composition. Such approaches provide deeper insight into the biological roles and cellular origins of EV populations.

Several analytical techniques have been developed to enable single-vesicle characterization. These include nanoparticle tracking analysis (NTA), high-resolution flow cytometry, nano-flow cytometry, and imaging flow cytometry (IFCM)[76, 109, 110]. Each of these methods provides complementary information about EV populations and contributes to the expanding analytical toolbox available for EV research. Nanoparticle tracking analysis is widely used for determining the size distribution and concentration of EVs in biological samples. This technique measures the Brownian motion of particles suspended in solution and calculates particle size based on their movement[111]. While NTA provides valuable quantitative information regarding particle size and abundance, it does not enable detailed characterization of vesicle surface markers.

High-resolution flow cytometry has also been adapted for EV analysis. Conventional flow cytometers were originally designed to analyse cells, which are considerably larger than EVs. Consequently, early cytometry platforms had limited sensitivity for detecting nanoscale

particles[112]. Recent advances in optical detection systems and fluorescence sensitivity have significantly improved the ability of modern instruments to analyse small particles such as EVs labelled with fluorescent antibodies[109, 112]. Nano-flow cytometry represents another technological advancement specifically optimized for nanoscale particle analysis[110]. This method combines principles of traditional flow cytometry with enhanced optical detection, enabling improved characterization of small vesicles and nanoparticles.

Among currently available single-vesicle technologies, IFCM represents a particularly powerful approach for EV analysis[113]. IFCM integrates the quantitative capabilities of flow cytometry with high-resolution microscopy, allowing simultaneous acquisition of fluorescence signals and brightfield or fluorescence images for each detected particle. This dual functionality enables both quantitative measurement of marker expression and visual confirmation of detected vesicles.

One of the major advantages of IFCM is the possibility to directly visualize fluorescently labelled vesicles[113, 114]. Because EVs are extremely small particles, distinguishing true vesicle signals from background noise, antibody aggregates, or other artifacts can be challenging. The imaging component of IFCM provides an additional level of validation by allowing researchers to confirm the presence and morphology of individual particles. In addition to vesicle detection, IFCM enables detailed phenotypic characterization of EV populations. By labelling vesicles with antibodies targeting specific surface proteins, researchers can identify EVs originating from particular cell types[114]. For example, vesicles expressing endothelial markers, immune cell markers, or epithelial markers can be distinguished based on their fluorescence profiles. This capability is particularly valuable when analysing EVs present in complex biological fluids, where vesicles may originate from numerous tissues and cell types. Identifying specific EV subsets can therefore provide important insights into the cellular sources contributing to EV populations observed in different disease states. Another advantage of IFCM is its relatively high throughput, which allows analysis of large numbers of vesicles within short acquisition times. This feature enables the detection of rare EV populations that might remain undetected using lower-throughput analytical methods.

The development of single-vesicle technologies has significantly expanded the possibilities for studying EV heterogeneity and identifying disease-associated vesicle populations[17, 76, 109]. By combining single-vesicle phenotyping with bulk molecular approaches such as proteomics, researchers can obtain a more comprehensive understanding

of EV biology[76]. In complex diseases such as endometriosis, where multiple cell types contribute to the pathological microenvironment, analysis of EV subpopulations may provide important insight into intercellular communication and signalling pathways. Vesicles derived from immune cells, endothelial cells, or endometrial cells may carry distinct molecular signatures reflecting their specific biological functions. Integrating single-vesicle phenotyping with molecular profiling therefore represents a powerful strategy for investigating EVs in disease contexts. While proteomic analysis can reveal global patterns of protein expression within EV populations, single-vesicle analysis enables identification of specific vesicle subsets contributing to these molecular signals.

Together, these complementary approaches provide a more complete picture of EV composition and function. Understanding both the molecular cargo and cellular origin of EVs is essential for interpreting their roles in physiological and pathological processes.

1.10. Biological rationale for studying EVs in plasma and PF

Endometriosis develops primarily within the peritoneal cavity, where ectopic endometrial lesions interact with surrounding tissues and immune cells[1, 9]. The peritoneal environment represents a complex biological niche characterized by chronic inflammation, immune cell infiltration, angiogenesis, and extensive tissue remodelling[8, 9]. Within this microenvironment, numerous molecular mediators contribute to the establishment, persistence, and progression of endometriotic lesions.

PF plays a particularly key role in this context. This fluid bathes the organs of the pelvic cavity and serves as a medium through which cells, cytokines, and signalling molecules interact[8]. In women with endometriosis, the composition of PF is frequently altered compared with individuals without the disease. Increased concentrations of inflammatory cytokines, growth factors, and immune mediators have been detected in this compartment, reflecting the presence of a persistent inflammatory microenvironment[8, 23]. Because PF is located in direct proximity to endometriotic lesions, it represents a valuable source of information about local biological processes associated with the disease[8]. Molecules present in PF may originate from multiple cellular sources, including ectopic endometrial cells, immune cells, stromal cells, and endothelial cells. Consequently, analysis of PF provides insight into the signalling networks operating within the peritoneal cavity.

EVs present in PF may carry molecular cargo derived directly from cells within the lesion microenvironment[89]. Vesicles released by ectopic endometrial cells may contain

proteins and nucleic acids reflecting pathological processes occurring within lesions. Similarly, EVs derived from immune or stromal cells may reflect inflammatory responses and tissue remodelling events associated with disease progression.

Because EVs function as mediators of intercellular communication, vesicle populations present in PF may participate in the dynamic interactions between different cell types within the endometriotic microenvironment[89, 115]. Through the transfer of bioactive molecules, EVs may influence immune responses, angiogenesis, cellular proliferation, and ECM remodelling.

While PF provides valuable information about local processes occurring in the pelvic cavity, systemic biological fluids may also contain molecular signals associated with endometriosis. Plasma represents one of the most accessible biological fluids for clinical investigations and has therefore been widely studied in the search for non-invasive disease biomarkers[70, 71, 116]. Circulating EVs present in plasma originate from multiple tissues throughout the body[115]. These vesicles may carry molecular cargo reflecting systemic responses to disease, including inflammatory signalling pathways and immune system activation. In the context of endometriosis, plasma-derived EVs may therefore provide insight into systemic alterations associated with the disease.

The analysis of EVs in plasma offers several practical advantages for biomarker discovery. Blood collection is minimally invasive, relatively simple to perform, and can be repeated during clinical follow-up[71, 116]. As a result, plasma-derived EVs represent an attractive source of molecular markers that could potentially be used for disease detection or monitoring. However, EV populations present in plasma are typically more heterogeneous than those observed in localized biological compartments such as PF[115]. Because circulating vesicles originate from numerous tissues, identifying disease-specific molecular signals within plasma EV populations can be challenging. For this reason, comparative analyses between EV populations derived from local and systemic compartments may provide particularly valuable insights.

Studying EVs derived from both PF and plasma makes it possible to investigate the relationship between local disease processes and systemic molecular responses. EV populations present in PF may primarily reflect the microenvironment of endometriotic lesions, whereas plasma-derived EVs may capture broader systemic alterations associated with the disease. Comparative analysis of EV cargo between these compartments may therefore reveal molecular signatures shared between local and systemic environments. Such

signatures could represent promising candidates for non-invasive biomarkers, as they would be detectable in easily accessible blood while still reflecting pathological processes occurring within the pelvic cavity[70, 71, 116].

The integration of EV research with high-throughput molecular technologies has further expanded the possibilities for studying these vesicles in complex diseases[17]. Proteomic profiling of EV populations enables large-scale identification and quantification of proteins present within vesicle cargo, allowing the identification of molecules differentially abundant between patient groups and the exploration of biological pathways associated with disease mechanisms[101].

In addition to bulk molecular profiling, single-vesicle analysis techniques provide complementary information about EV heterogeneity and cellular origin[117]. Methods such as IFCM allow detailed characterization of vesicle surface markers and enable identification of specific EV subpopulations within complex biological samples[118]. Combining these analytical approaches provides a comprehensive strategy for investigating EVs in disease contexts. While proteomic analysis can reveal global molecular signatures associated with disease, single-vesicle phenotyping enables identification of specific vesicle populations contributing to these molecular patterns.

In the context of endometriosis, such an integrated approach may help uncover molecular pathways involved in disease development and facilitate the identification of candidate biomarkers. By comparing EV populations derived from PF and plasma, it becomes possible to explore both local and systemic aspects of disease biology. Understanding how EV-mediated communication contributes to the interaction between endometrial cells, immune cells, and other components of the peritoneal microenvironment may provide important insights into the mechanisms underlying endometriosis. At the same time, identifying molecular signatures present in circulating EV populations may support the development of minimally invasive diagnostic strategies.

For these reasons, the investigation of EVs derived from both PF and plasma represents a promising strategy for studying the molecular landscape of endometriosis. Integrating proteomic analysis with single-vesicle phenotyping technologies provides a powerful framework for characterizing EV populations and exploring their potential as sources of disease-associated biomarkers.

2. ASSUMPTIONS AND OBJECTIVES OF THE STUDY

2.1. Assumptions of the study

Endometriosis remains a chronic gynaecological disease for which reliable, non-invasive biomarkers suitable for routine clinical application are still lacking. Despite extensive research efforts, currently available diagnostic strategies rely predominantly on invasive procedures, including laparoscopy, while so far proposed circulating biomarkers have demonstrated insufficient sensitivity and specificity for clinical implementation.

It is further assumed that EVs represent a biologically meaningful and stable source of molecular information, reflecting both local pathological processes occurring in the peritoneal environment as well as systemic alterations detectable in peripheral circulation. The molecular cargo of EVs, particularly proteins, is thought to mirror disease-associated mechanisms such as immune dysregulation, chronic inflammation, oxidative stress, ECM remodelling, and altered cell-cell communication.

The study assumes that high-resolution proteomic analysis enables the discovery of EV-associated molecular signatures relevant to endometriosis pathophysiology. At the same time, it acknowledges that proteomics, while powerful for discovery, is costly and poorly suited for routine clinical implementation. Therefore, it is assumed that identifying EV-associated protein markers may provide a foundation for subsequent validation using more scalable and clinically applicable techniques, such as cytometry-based EV phenotyping.

2.2. Aim of the study

Main aim

The main aim of this doctoral thesis was to characterize and compare the proteomic profiles of EVs isolated from PF and plasma of patients with endometriosis and disease-free controls, and to evaluate their significance in reflecting local and systemic mechanisms involved in endometriosis progression.

Specific aims

The specific aims of the study were to:

1. Isolate and comprehensively characterize EVs derived from PF and plasma of women with surgically confirmed endometriosis and control subjects.
2. Perform high-resolution, TMT-based proteomic analysis of EVs to identify EV-associated DEPs distinguishing endometriosis patients from controls.
3. Compare EV proteomic signatures between PF and plasma, in order to differentiate local disease-specific molecular alterations from systemic manifestations of endometriosis.
4. Identify biological pathways and functional networks linked to EV-associated DEPs that may be relevant to endometriosis progression.
5. Define a subset of EV-associated proteins shared between PF and plasma, potentially representing systemically accessible biomarkers reflecting peritoneal disease activity.
6. Assess the translational potential of EV-based proteomic findings, providing a rationale for future development of non-invasive, high-throughput diagnostic approaches, including cytometry-based EV phenotyping.

3. MATERIALS AND METHODS

3.1. Study design and patient recruitment

Biological material, including plasma and PF, was collected from patients with endometriosis and controls as part of a multicentre, cross-sectional study conducted across eight Departments of Obstetrics and Gynaecology in Poland between 2018 and 2019 and funded by the Polish Ministry of Health (project number: 6/6/4/1/NPZ/2017/1210/13522).

The study, approved by the Ethics Committee of the Medical University of Warsaw (KB/223/2017), with written informed consent obtained from all participants, included women aged 18-40 years who were scheduled for laparoscopic procedures due to non-malignant conditions such as infertility, chronic pelvic pain syndrome, ovarian cysts, or suspected endometriosis. Exclusion criteria included prior pelvic surgery, suspected malignancy, current or past pelvic inflammatory disease, autoimmune disorders, hormone therapy within the preceding three months, uterine fibroids, polycystic ovaries, and irregular menstrual cycles (less than 25 days or more than 35 days). After applying these criteria and excluding outlier results, the final analysis included a total of 52 samples were included in the final analysis. This consisted of 26 plasma samples and 26 PF samples, with each group comprising 10 samples from patients diagnosed with endometriosis and 16 samples from disease-free controls.

Patients underwent laparoscopic evaluation, during which eventual endometrial lesions were classified according to the revised criteria of the American Society for Reproductive Medicine (rASRM). The diagnosis was further confirmed through histological examination of biopsy samples. Endometriosis patients were categorized into four stage subgroups (I-IV). Given the similarities in disease activity, patients with stage I and II endometriosis were categorized as having "mild" disease, while those with stage III and IV were classified as having "severe" disease. For this study, the research group included only patients with stage III and IV endometriosis, classified as severe disease, with a single patient classified as stage IV. The analysis focused on advanced cases characterized by a higher symptom burden and more extensive lesions. By investigating the proteome of EVs in this group, this study aims to identify distinct molecular signatures and biomarkers, providing insights into disease progression and uncovering potential targets for improved diagnosis and treatment. The control group included patients with no visible endometriosis lesions during laparoscopy. The phase of the menstrual cycle was determined based on the last menstrual

period and the average cycle length and further validated by histological evaluation of eutopic endometrial samples obtained during surgery. Detailed characteristics of the study population are presented in Table 1.

Table 1. Characteristics of study groups: endometriosis stage and menstrual cycle parameters. Comparison of the endometriosis group (rASRM stages III-IV) and the control group (C). Data include participants' age, clinical characteristics, and menstrual cycle parameters (cycle day and phase). "N/A" indicates data not applicable for the control group

Group	Age	Endometriosis Classification (rASRM)	Manifestation of the condition	Cycle Day	Cycle phase
Endometriosis group	28	III	Moderate Many deep implants Small cysts on one or both ovaries Presence of filmy adhesions	7	Proliferative
	26			11	
	37			12	
	28			8	
	32			11	
	30			11	
	29			10	
	33			10	
	39			10	
	30	IV	Severe Many deep implants Large cysts on one or both ovaries Many dense adhesions	10	
Control group	30	C	N/A	9	
	33			6	
	32			11	
	32			10	
	39			13	
	35			13	
	21			8	
	40			9	
	32			10	
	29			11	
	36			14	
	35			13	
	40			13	
	30			16	
	21			5	
	19			5	

3.2. Plasma and PF sample collection and processing

Biological material, including blood and PF, was collected in accordance with the standard operating procedures of the Endometriosis Phenome and Biobanking Harmonization Project (EPHect), developed by the World Endometriosis Research Foundation (WERF)[119]. The details of patient recruitment and sample collection have been described previously in a

multicentre cross-sectional study[120]. Briefly, blood samples were drawn into 10 mL ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) prior to surgery and before anaesthesia induction. PF was aspirated at the beginning of laparoscopy using a Veress needle under direct visual control to minimize the risk of blood contamination and ensure sample purity. The collected blood samples were centrifuged at 2,500 × g for 10 min at 4 °C to separate plasma, while PF samples were centrifuged at 1,000 × g for 10 min at 4 °C. After centrifugation, the resulting plasma and PF supernatants were carefully aliquoted into 500 µL tubes to facilitate subsequent analyses and stored at −80 °C. Consistent protocols for sample handling were followed at all participating centres to ensure the comparability of results. Furthermore, the time between sample collection and processing was carefully monitored and consistently kept under 45 min. The collection process did not interfere with the patients' medical management and was conducted in full compliance with the Declaration of Helsinki. All samples were transported on dry ice, first to the Department of Obstetrics and Gynaecology and subsequently to the Department of Biochemistry at the Medical University of Warsaw, where both plasma and PF samples were analysed.

3.3. Perioperative plasma cohort used for IFCM assay validation

An independent perioperative cohort was used to optimize and validate the IFCM assay applied for EV phenotyping. Plasma samples were obtained from patients with endometriosis before and 48 hours after laparoscopic surgical treatment. These samples were originally collected within the framework of our previously published study investigating perioperative molecular alterations in endometriosis[121]. Briefly, paired plasma samples were collected from 15 patients immediately before surgery and 48 hours after laparoscopic removal of endometriotic lesions. Sample collection, clinical characterization of patients, and initial plasma preparation were performed according to the procedures described in the original publication[121]. This cohort served for assay optimization, enabling assessment of the sensitivity and technical robustness of the IFCM-based EV phenotyping approach, as well as verification of the antibody panel used for EV surface marker detection.

3.4. Cell lines and cell culture.

To complement the proteomic analysis of EVs derived from plasma and PF, EVs and whole-cell lysates were obtained from relevant human cell lines. The human endometriotic epithelial

cell line 12Z (Applied Biological Materials Inc., Richmond, BC, Canada), which exhibits characteristics of endometriotic epithelial cells, and the ovarian endometrioid adenocarcinoma cell line MDAH-2774 (ATCC, USA), an established ovarian adenocarcinoma model, were selected for this study.

3.4.1 Cell culture conditions

12Z cells were cultured on Applied Cell extracellular matrix (Applied Biological Materials Inc., Canada) in PriGrow III medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (P/S; Sigma-Aldrich, Austria). Cells were seeded at a density of 1.8×10^4 cells/cm² and passaged every 3-4 days.

MDAH-2774 cells were maintained in DMEM supplemented with 2 mM L-glutamine (Sigma-Aldrich, Austria), 10% FBS, and 1% P/S. Cells were seeded at 1×10^4 cells/cm² and passaged every 4-5 days. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were washed with phosphate-buffered saline (PBS; Gibco, UK) and detached using 0.25% trypsin-EDTA (Gibco, UK) prior to passaging.

3.4.2 Collection of conditioned media

For EV isolation, cells were seeded in T175 flasks and grown to approximately 80% confluence. Cells were washed with PBS and cultured for 48 h in their respective media supplemented with exosome-depleted FBS (Thermo Fisher Scientific, USA). Conditioned media (50 mL per flask) were collected and centrifuged at 3,234 × g for 15 min at room temperature (RT) to remove cell debris. The supernatant was filtered through a 0.22 µm PES membrane filter (Bionovo, Poland) and stored at −80°C until further processing. Cells were harvested and counted using a Bürker chamber.

3.4.3 Concentration of conditioned media

Conditioned media were thawed overnight at 4°C and concentrated by centrifugation at 2,000 × g using 100 kDa molecular weight cut-off centrifugal filters (Vivacell 100, Sartorius, UK; or Amicon Ultra, Merck, Germany) to a final volume of 1 mL. When necessary, PBS was added to adjust the final volume to 1 mL prior to further EV isolation steps.

3.5. Isolation of EVs

EVs were isolated from biological matrices, including plasma, PF, and conditioned cell culture medium, sing size exclusion chromatography (SEC). Prior to SEC isolation, plasma and PF samples were pre-cleared by sequential centrifugation to remove cells and debris, as described above. Plasma and PF samples were centrifuged at 1,500 × g for 10 min, followed by 10,000 × g for 10 min to further clarify the supernatants. Conditioned cell culture medium was processed separately as described in the previous section.

For proteomic and IFCM analyses, commercially available qEV Original Gen 2 (70 nm cutoff) SEC columns (Izon Science, Christchurch, New Zealand) were used according to the manufacturer's protocol. These columns were selected due to their higher reproducibility, improved EV recovery, and more efficient removal of contaminating plasma proteins and non-vesicular particles, which is critical for MS-based protein profiling. Due to column capacity limitations, a maximum input volume of 0.5 mL per sample was applied. A total of 0.5 mL of sample was loaded onto the column. After the sample entered the column bed, buffer was added and allowed to pass through the column, corresponding to the void volume. Following the elution of 2.9 mL of buffer (void volume), EV-containing fractions were collected, and four consecutive fractions of 0.4 mL each (fractions 8-11) were pooled for further analyses.

For other downstream applications, laboratory-prepared SEC columns packed with Sepharose CL-2B (GE Healthcare, USA) were used, as previously described[122], with minor modifications. A total of 1 mL of sample (plasma or PF) was loaded onto the column. After the sample entered the column bed, 2 mL of PBS were added sequentially (2 × 1 mL) and allowed to pass through the column, corresponding to the void volume. Following the elution of the void volume, EV-containing fractions were collected. Specifically, two consecutive fractions of 1 mL each (fractions 4 and 5) were collected into Eppendorf tubes and considered EV-enriched.

These fractions were subsequently analysed using NTA and a bicinchoninic acid (BCA) protein assay (Pierce™, Thermo Fisher Scientific, Waltham, MA, USA; cat. no. 23227), according to the manufacturer's instructions. The validated fractions were then pooled into a final volume of 2 mL and subsequently concentrated using Amicon® Ultra-2 mL centrifugal filters (Merck, Darmstadt, Germany) at 4,000 × g for approximately 30 min at RT, and stored at −80 °C until further use.

3.6. NTA analysis of EVs

Prior to staining, predilution of EV samples, antibodies, or dyes was performed as needed and all EV samples were measured in scatter mode to determine particle concentration. Samples were diluted in PBS without Ca^{2+} and Mg^{2+} (Lonza, Basel, Switzerland) to approximately 350 particles per frame, which was chosen as the optimal concentration for labelling experiments. The predilution of EV samples was adjusted as needed to achieve the highest concentration within the optimal range for scatter mode measurements after the final post-labelling dilution, and it varied according to the original concentration of each EV sample. Scatter mode measurements were conducted at RT across 11 positions in two cycles using the following settings for plasma- and PF-derived EVs: Sensitivity: 80, Shutter: 100, Minimal Brightness: 30, Trace length: 15, Min Area: 10, Max Area: 1000 nm/Class: 5, Classes/Decade: 64, Resolution: medium.

To distinguish non-lipid particles from EVs, the samples were stained with the lipid membrane-specific dye CellMask™ Deep Red (CMDR, Thermo Fisher Scientific, Waltham, MA, USA) and analysed using fluorescent nanoparticle tracking analysis (F-NTA). The optimal CMDR concentration for F-NTA, ensuring maximal EV staining with minimal background (CMDR in PBS), was determined to be 4 ng/mL (see Table 2). For immunolabelling, fluorescence-labelled antibodies targeting tetraspanins (CD63, CD9 and CD81), which are typical EV markers, were used (see Table 2). The highest antibody concentration without significant background fluorescence was selected as the optimal final dilution for F-NTA measurements.

Table 2. Fluorescent dyes and antibodies used for F-NTA analysis.

Antibody/dye	Source, Catalog number:	Antigen	Dilution	Application
CellMask™ Deep Red plasma membrane stain	Invitrogen™ Thermo Fisher Scientific, C10046	Membrane Stain	1:1250000	F-NTA
PE	Invitrogen™ Thermo Fisher Scientific, MA1-19650	CD63	1:5000	F-NTA
PE	Biolegend, 349506	CD81	1:40000	F-NTA
PE	Biolegend, 312106	CD9	1:6000	F-NTA

Fluorescence labelling was performed using prediluted EVs and prediluted antibody/CMDR at an approximately 9:1 ratio in a total volume of 10–50 µL for 2 h at RT in the dark.

Subsequently, EVs were further diluted in sterile 1× PBS (without Ca^{2+} or Mg^{2+}) at a 1:1000 ratio and analysed using F-NTA at RT across 11 positions in one cycle.

The following settings were used for antibody staining: F488, sensitivity 95, shutter 100, minimal brightness 25, trace length 7, minimum area 10, maximum area 1000, nm/class 5, classes/decade 64, resolution medium. For CMDR staining, the settings were: F640, sensitivity 91, shutter 100, minimal brightness 25, trace length 7, minimum area 10, maximum area 1000, nm/class 5, classes/decade 64, resolution medium. Each immunolabelled sample was first evaluated in fluorescence mode with the "low bleach" function activated, followed immediately by assessment in scatter mode to minimize photobleaching. A minimum of three measurements was performed per sample. To ensure day-to-day repeatability and precision of size and concentration measurements in scatter mode, daily measurements of 100 nm polystyrene beads (PS100; Particle Metrix GmbH, Inning am Ammersee, Germany) were performed, and the coefficient of variation was calculated.

3.7. Determining EV protein concentration by BCA assay

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Bovine serum albumin (BSA) standards ranging from 0-1,000 µg/mL were prepared in water. Concentrated EV samples were diluted 1:10 in water prior to analysis, whereas EV-rich fractions obtained from SEC were analysed without additional dilution. Aliquots of 25 µL of standards, samples, and blank (25 µL H_2O) were loaded in triplicate into a flat-bottom 96-well microplate. Subsequently, 200 µL of working reagent containing BCA Reagent A (carbonate buffer) and Reagent B (4% cupric sulfate solution) mixed at a 50:1 ratio was added to each well. The plate was incubated for 30 min at 37°C with shaking. Absorbance was measured at 562 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). Protein concentrations were calculated based on the BSA standard curve.

3.8. Western blotting

3.8.1 Sample preparation

EV samples were lysed in RadioImmunoPrecipitation Assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) to prevent proteolytic degradation during sample preparation. Lysis was

performed on ice to preserve protein integrity. Following protein quantification by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA), equal amounts of total EV protein (10 µg per lane) were prepared for electrophoretic separation. Samples were mixed with 5× reducing Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing β-mercaptoethanol. The final concentration of the sample buffer was adjusted to 1× prior to loading. The mixtures were heated at 95°C for 5 min to ensure complete protein denaturation and reduction of disulfide bonds, thereby facilitating linearization of proteins and uniform migration during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After heat treatment, samples were briefly centrifuged to collect condensation and remove air bubbles before being loaded onto polyacrylamide gels for electrophoretic separation.

3.8.2 Preparation of polyacrylamide gels

Polyacrylamide gels (1.5 mm thickness) were prepared for SDS-PAGE using a Mini-PROTEAN system (Bio-Rad Laboratories, Hercules, CA, USA). Resolving gels (12% acrylamide) and stacking gels were formulated according to the compositions listed in Table 3. After polymerization of the resolving layer, the stacking gel was poured on top to complete the gel assembly.

Table 3. Reagents and volumes used for the preparation of polyacrylamide gels.

Resolving gel						
Gel %	H_2O (mL)	30% acrylamide (mL)	1.5 M Tris-HCl, pH 8.8 (mL)	10% SDS (µL)	10% APS (µL)	TEMED (µL)
12%	3.2	4.0	2.6	100	100	10
Stacking gel						
Gel %	H_2O (mL)	30% acrylamide (mL)	1.5 M Tris-HCl, pH 6.8 (mL)	10% SDS (µL)	10% APS (µL)	TEMED (µL)
4%	5.86	1.34	2.6	100	100	10

Abbreviations used in the table: SDS, sodium dodecyl sulfate; APS, ammonium persulfate; TEMED, N,N,N′,N′-tetramethylethylenediamine.

3.8.3 SDS-PAGE

Equal amounts of protein (10 µg per lane) were loaded onto denaturing polyacrylamide gels and separated by SDS-PAGE. Electrophoresis was performed using a ProteanPlus™ Tetra Cell system (Bio-Rad Laboratories, Hercules, CA, USA) in running buffer consisting of 25

mM Tris-HCl, 192 mM glycine, and 0.1% SDS. A current of 30 mA was applied during protein migration through the stacking gel and increased to 40 mA for separation within the resolving gel.

3.8.4 Semi-dry electrotransfer

After electrophoresis, proteins were transferred from the gel onto a 0.2 µm nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) using a Trans-Blot® Turbo™ semi-dry transfer system (Bio-Rad Laboratories) with a custom transfer program (1 A, 25 V, 50 min). The transfer sandwich was assembled in dry transfer cassettes, with filter papers moistened with transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol) placed on both sides of the gel and membrane, enabling efficient protein transfer under semi-dry conditions. To verify the transfer efficiency, membranes were briefly stained with Ponceau S solution (Abcam). Ponceau S is a reversible dye that interacts electrostatically with positively charged amino groups and associates non-covalently with hydrophobic regions of proteins. Membranes were incubated in the staining solution for 5 min with gentle agitation, followed by rinsing in Tris-buffered saline with Tween-20 (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) until protein bands became clearly visible. The membranes were subsequently washed in TBST until complete destaining was achieved.

3.8.5 Immunoblotting and chemiluminescent detection

After transfer, membranes were blocked for 1 h at RT in 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) prepared in TBST, with gentle agitation. Membranes were subsequently incubated overnight at 4 °C with primary antibodies against Calnexin, EHD4, Tsg101, CD9, or CD81 (Table 4). Following three washes in TBST (10 min each), membranes were incubated for 2 h at RT with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 4) under constant agitation. Membranes were then washed three times with 1× TBST (10 min each). Immunoreactive bands were visualized using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions. The substrate components were mixed at a 1:1 ratio and applied to the membranes for 2 min prior to signal acquisition. Chemiluminescent signals were detected using a ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA, USA) and quantified with Image Lab software (Bio-Rad).

Table 4. Primary and secondary antibodies used for Western blot analysis.

Antibody	Source, catalogue number:	Antigen	Dilution	Type
Anti-Calnexin	abcam, ab13504	Calnexin	1:2000	Primary
Anti-EHD4	abcam, ab 151694	EHDR	1:2000	Primary
Anti-Tsg-101	abcam, ab30871	TSG-101	1:1000	Primary
Anti-CD81	Invitrogen™ Thermo Fisher Scientific, 10630D	CD81	1:1000	Primary
Anti-CD9	Invitrogen™ Thermo Fisher Scientific, 10626D	CD9	1:500	Primary
HRP	Thermo Fisher Scientific, A16078	mouse antibodies	1:10000	Secondary
HRP	Thermo Fisher Scientific, A16110	rabbit antibodies	1:10000	Secondary

3.9 Immunophenotyping of EVs by IFCM

EVs were analysed at the single-vesicle level using IFCM on an AMNIS ImageStreamX Mark II platform (AMNIS/Luminex, Seattle, WA, USA). Analyses were performed either on isolated EV preparations or directly in native plasma samples. The measurements were conducted at the Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Germany. Instrument configuration and acquisition parameters were adapted from previously optimized protocols for small EV detection[114, 123, 124].

3.9.1 Methodological strategy

To establish a robust staining protocol, initial optimization experiments were performed using EVs isolated from plasma in order to reduce background signals originating from non-vesicular plasma components. After optimization of antibody concentrations, dilution ranges, and acquisition parameters, the protocol was subsequently applied directly to native plasma samples. This stepwise strategy enabled validation of the staining and acquisition workflow under reduced-background conditions prior to its implementation in a clinically relevant biological matrix. Direct analysis of plasma samples was pursued to minimize EV loss associated with isolation procedures and to enhance the translational applicability of the approach.

3.9.2 Preparation of antibody solutions

Fluorochrome-conjugated anti-human monoclonal antibodies targeting 25 different markers (Table 5) were diluted in sterile, filtered PBS. To remove potential antibody aggregates and fluorochrome precipitates that could generate false-positive signals during single-EV acquisition, diluted antibody solutions were centrifuged at 17,000 × g for 10 min at 4 °C. The supernatant was carefully transferred to fresh low-binding tubes and protected from light until use.

Table 5. Antibody panel used for EV immunophenotyping by IFCM.

Antibody	Clone	Catalog No.	Manufacturer
CD9-PE	MEM-61	1P-208-T100	Exbio, Vestec, Czech Republic
HLA-ABC-FITC	B9.12.1	IM1838U	Beckman Coulter, Indianapolis, IN, USA
HLA-DR-ECD	Immu-357	B92438	Beckman Coulter, Indianapolis, IN, USA
CD82-PE	ASL-24	342104	BioLegend, San Diego, CA, USA
CD14-APC	REA599	130-110-520	Miltenyi Biotec, Bergisch Gladbach, Germany
CD44-APC	MEM-85	1A-221-T100	Exbio, Vestec, Czech Republic
CD81-FITC	JS64	B25329	Beckman Coulter, Indianapolis, IN, USA
CD90-APC	5E10	1A-652-T100	Exbio, Vestec, Czech Republic
CD227-FITC	HMPV	559774	BD Pharmingen
CD31–BV421	WM-59	303124	BioLegend, San Diego, CA, USA

3.9.3 EV staining procedure

Surface expression of EV-associated markers was assessed using fluorochrome-conjugated monoclonal antibodies listed in Table 5. Plasma samples were thawed and centrifuged at 10,000 × g for 10 min at RT to remove residual debris. Subsequently, 10 µL of the plasma supernatant or isolated EV preparations were incubated for 2 h at RT in the dark with previously titrated optimal antibody dilutions. After incubation, samples were diluted in filtered PBS prior to acquisition to reduce background fluorescence from unbound antibodies and to ensure measurements within the linear detection range of the instrument (10^6-10^8 events/mL). To minimize coincident particle detection (swarm effect), samples were further serially diluted prior to measurement.

3.9.4 Control conditions

To validate analytical specificity and control for background signals, the following controls were included:

- Buffer control (PBS only)
- Antibody-only control (antibody without EVs)
- Unstained sample
- Detergent-treated sample

All controls were processed identically to experimental samples, except for the omitted component.

3.9.5 Detergent lysis control

To confirm the vesicular origin of detected fluorescent events, detergent lysis controls were performed using 1% Nonidet P-40 (NP-40; Sigma-Aldrich, St. Louis, MO, USA), a non-ionic detergent that disrupts lipid membranes. Stained samples were incubated with NP-40 for 30 min at RT prior to acquisition. A marked reduction in fluorescent events after detergent treatment was interpreted as confirmation of membrane-dependent signal detection.

3.9.6 Acquisition parameters and swarm control

All samples were diluted 50-500-fold in PBS prior to acquisition. Data were acquired using an ImageStreamX Mark II imaging flow cytometer (AMNIS/Luminex, Seattle, WA, USA) with fluorescence-triggered detection.

To minimize coincident (swarm) particle detection, serial dilution was performed to ensure acquisition within the linear detection range. Samples and controls were acquired for 3-5 min. Forward scatter was disabled, and fluorescence signals were collected using all available laser lines. Fluorescence calibration and reporting in molecules of equivalent soluble fluorophore (MESF) units were performed as previously described[114, 123]. Dulbecco's PBS (pH 7.4; Gibco, Thermo Fisher Scientific, Waltham, MA, USA)was used as sheath fluid.

3.10. Cryogenic Electron Microscopy (Cryo-EM)

Direct visualization of EVs was performed using cryo-EM. A total of 3 μL of concentrated EV samples, derived from both plasma and PF of a single endometriosis patient, were plunge-frozen onto glow-discharged Quantifoil Au R1.2/1.3 holey carbon grids with 2 nm ultrathin carbon (Quantifoil Micro Tools GmbH, Jena, Germany) using a Vitrobot Mark IV (Thermo

Fisher Scientific, Waltham, MA, USA) with the following settings: blot time 4 s, blot force 4, and waiting time 10 s.

Two-dimensional cryo-electron microscopy images were acquired using a Glacios transmission electron microscope (Thermo Fisher Scientific, Waltham, MA, USA) operating at 200 kV, equipped with a Falcon 3EC direct electron detection camera (Thermo Fisher Scientific) at a nominal magnification of 92,000×, corresponding to a pixel size of 1.587 Å at the specimen level. The defocus was set to 3 µm, and the total electron dose was approximately 50 e/Å^2.

3.11. Protein identification and quantitation of EVs using TMT-based MS

3.11.1 Proteomics sample preparation

Isolated EVs and cells in 50 µL of PBS were supplemented to a total volume of 100 µL with 100 mM triethylammonium bicarbonate (TEAB) buffer. SDS was added to the samples to a final concentration of 1%. The samples were vortexed for 30 min, sonicated in an ultrasonic bath for 30 min, and incubated at 95 °C for 10 min. Following lysis, the material was centrifuged for 30 min at 21,000 × g at 20 °C. Protein concentration in the samples was measured using the BCA method (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's instruction. Internal standards (IS) for the analysis of vesicles from plasma and PF were prepared by pooling small amounts of each sample within a given experiment. Additionally, to enhance the detection of non-plasma-derived proteins, mixed samples of MDAH and 12Z cells (cell proteome, CP), as well as EVs derived from their cultures (cell-derived EVs, CD-EVs), were prepared. Detailed information regarding cell lines, cell culture, and the collection of conditioned media for CD-EV isolation is provided in Sections 3.4–3.5. For digestion, 40 µg of protein from each sample and 80 µg of each IS, CP, and CD-EV was transferred to a new low retention tube. After 1 h incubation at 60 °C with 10 mM tris(2-carboxyethyl)phosphine (TCEP), cysteines were blocked by a 30-minute reaction with 30 mM chloroacetamide. Samples were digested using a modified Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) method with minor adjustments. The magnetic bead mix was prepared by combining equal parts of Sera-Mag Carboxyl hydrophilic and hydrophobic particles (65152105050250 and 45152105050250, Cytiva, Marlborough, Massachusetts, USA). The bead mix was washed three times with mass spectrometry–grade (MS-grade) water and resuspended to a working concentration of 10 µg/µl.

40 µl of the bead mix and 1% formic acid (FA) in acetonitrile (ACN) were added to bring the final ACN concentration to 82%. Proteins were bound to the beads by incubating the mixture on a vortex for 30 min, followed by three washes with 85% ethanol and a final wash with acetonitrile. 2 µg of trypsin (Promega, Walldorf, Germany) in 100 µl of 100 mM TEAB was added to each sample, and digestion was carried out overnight at 37 °C on a vortex. The resulting peptide solutions were transferred to new tubes, the resin was further washed with 40 µl of 1% dimethyl sulfoxide (DMSO) in water, followed by 60 µl of water. Peptide aliquots were dried in SpeedVac and resuspended in 80 µl of 100 mM TEAB. 40 µl of peptide solution, corresponding to 20 µg of peptides from samples and IS, and 10 µg of peptides from CP and CD-EV samples was labelled with 0.25 mg TMTpro 16-plex reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reconstituted in 40 µl of acetonitrile. For each type of EVs (isolated from plasma or PF), two TMT sets were prepared. Tag 126 was used to label IS samples, while tags 133C and 134N were used to label CD-EV and CP samples, respectively. The reaction was quenched by the addition of 8 µL 5% hydroxylamine. After checking the labelling efficiency, peptides were combined within each TMTpro set and desalted using 30 mg Oasis HLB columns (Waters, Milford, Massachusetts, USA). Briefly, cartridges were preconditioned with 1 ml methanol and washed with 1 ml 1.5% ACN and 0.1% FA. After sample loading and rinsing with 1 ml 1.5% ACN and 0.1% FA, peptides were eluted from columns with 400 µl 90% ACN and 0.1% FA. Aliquots were dried in SpeedVac and resuspended in 500 µl 10 mM ammonium hydroxide with 2% ACN.

The mixed TMT-labelled peptides were separated using high-pH reverse-phase chromatography (HpRP) on an XBridge Peptide BEH C18 column (4.6 x 250 mm, 130 Å, 5 µm, Waters). The separation was conducted at a flow rate of 0.8 ml/min over a 50-minute gradient on a Waters Acquity UPLC H-class system (Milford, Massachusetts, USA). Mobile phases included water (A), acetonitrile (B), and 100 mM ammonium hydroxide (C), with phase C maintained at 10% throughout the gradient. Fraction collection started 2 min into the gradient, with one fraction collected every minute. The applied gradient was as follows: solvent B from 3% to 8% over the first 2 min, from 8% to 15% over 8 min, from 15% to 25% over 17 min, from 25% to 33% over 8 min, from 33% to 50% over 7 min, and then raised to 90% over 4.5 min. This was followed by a 1.5-min isocratic hold and a final equilibration at 3% solvent B for 2 min. The peptide elution was monitored at 214 nm. In total, 48 fractions were collected, dried in SpeedVac, and reconstituted in Evosep solvent A (0.1% FA in water)

with 30 min of vortexing followed by 30 min sonication. Every 24th fraction was concatenated, resulting in a final set of 24 fractions for each TMT set.

3.11.2 MS analysis

Fractions were analysed using an LC-MS system composed of an Evosep One (Evosep Biosystems, Odense, Denmark) directly coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Concatenated peptide fractions were loaded onto disposable Evotips C18 trap columns (Evosep Biosystems, Odense, Denmark) as described previously[125]. Chromatography was carried out at a flow rate of 1 µl/min using the 21 min (60 samples per day) preformed gradient on EV1109 analytical column (ReproSil Saphir C18 Dr Maisch, 1.5 µm beads, 150 µm ID, 8 cm long, Evosep Biosystems, Odense, Denmark). Data were acquired in positive mode with a data-dependent method with a 1 s cycle time using the following parameters: MS1 resolution was set at 60,000 with a normalized AGC target of 300% to ensure sufficient ion accumulation. Auto maximum inject time, and a scan range of 300 to 1700 m/z. MS2 resolution was set to 30,000, with a standard normalized AGC target, automatic maximum injection time, and an isolation window of 1.2 m/z. Precursor fragmentation was performed using higher-energy collisional dissociation (HCD) with a normalized collision energy of 30%. TurboTMT resolution mode was set to "TMTpro reagents." Spray voltage was set to 2.1 kV, funnel radio frequency (RF) level to 40, and heated capillary temperature to 275 °C.

3.11.3 Data processing and functional annotation

Offline recalibration, as well as peptide and protein identification, was performed using the MaxQuant/Andromeda software suite (version 2.0.1.0)[126] with the Homo sapiens Swissprot reference database (version 202306) and MaxQuant contaminants database. The search included tryptic peptides, carbamidomethylation (C) as a fixed modification, and oxidation (M) as a variable one. Reporter MS2 TMTpro 16-plex quantification was specified to obtain quantitative analysis values. A reverse database was used for target/decoy statistical validation, with peptide and protein false discovery rate (FDR) set to 1%. Protein groups and quantitative data were further analysed in Perseus (version 1.6.15)[127]. Channels corresponding to CP and CD-EV samples were excluded from further analysis. Reversed database hits, contaminants, proteins identified by site, or proteins identified in only one out of two TMT sets with fewer than two peptides were removed.

Data normalization used the modified loading factors (LF) method. LF coefficients for each TMTpro channel were calculated by dividing the median signal of all samples by the median signal of proteins within a given channel. Data were logarithmized, missing data were imputed using a shifted normal distribution, and batch effects were removed using the Limma R algorithm. Validity was confirmed through data distributions, Pearson correlation, and principal component analysis (PCA). Statistical testing was conducted using a Student's t-test with and without FDR correction.

Functional annotation and enrichment analyses were independently conducted by the lead author to further interpret the identified proteins. Protein functions were assigned using the UniProt Knowledgebase (UniProtKB; https://www.uniprot.org/, accessed on 31 January 2025), Gene Ontology (GO; http://geneontology.org, accessed on 31 January 2025), and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/, accessed on 31 January 2025). Enrichment analyses were performed using Functional Enrichment Analysis Tool (FunRich; version 3.1.4; standalone software; http://www.funrich.org/, accessed on 31 January 2025), ShinyGO (version 0.81; http://bioinformatics.sdstate.edu/go/, accessed on 31 January 2025), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; version 12.0; https://string-db.org/, accessed on 31 January 2025), Cytoscape (version 3.10.0; standalone software; https://cytoscape.org/, accessed on 31 January 2025), and Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://davidbioinformatics.nih.gov/, accessed on 31 January 2025). Heatmaps and hierarchical clustering analyses were performed using Morpheus (Broad Institute; https://software.broadinstitute.org/morpheus/, accessed on 31 January 2025).

3.12. Statistical analysis

Data analysis was performed using GraphPad Prism (version 8, GraphPad Software, San Diego, CA, USA). IFCM data were acquired using INSPIRE software (Luminex) and analysed using IDEAS software (Luminex).

Western blot data were analysed using Image Lab software (Bio-Rad). NTA data were initially processed using instrument-dedicated software (Particle Metrix GmbH) and subsequently analysed using FlowJo software (FlowJo, LLC). Data distribution was assessed using the Shapiro–Wilk test. For comparisons between two independent groups, an unpaired Student's t-test or Mann–Whitney U test was applied, depending on data normality. For paired comparisons (e.g., before and after surgery), paired t-test or Wilcoxon signed-rank test

was used. For comparisons involving more than two groups, one-way or two-way ANOVA followed by Tukey's multiple comparison test was applied.

Data are presented as mean ± standard deviation (SD) or median with interquartile range (IQR), as appropriate. Box plots were generated using Tukey's method (1.5×IQR). Outliers were identified and excluded prior to statistical analysis. A p-value < 0.05 was considered statistically significant. Statistical significance was indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4. RESULTS

4.1. Characterization of plasma and PF EVs

Plasma and PF contain high levels of albumin and other soluble proteins that may co-isolate with EVs during centrifugation-based methods, potentially interfering with downstream analyses, including proteomics. Therefore, EVs were isolated using SEC. This method is widely adopted for EV enrichment across diverse biological samples, as it removes the most contaminants and allows the purification of functionally active EVs.

In preliminary optimization experiments, self-made SEC columns were evaluated to assess their suitability for EV enrichment from plasma and PF samples of patients with endometriosis. These experiments validated the feasibility of this approach for EV isolation from both biological fluids. In Figure 4, the graph presents the distribution of EV particle concentration and total protein content across seven SEC fractions obtained from 1 mL aliquots of plasma and PF.

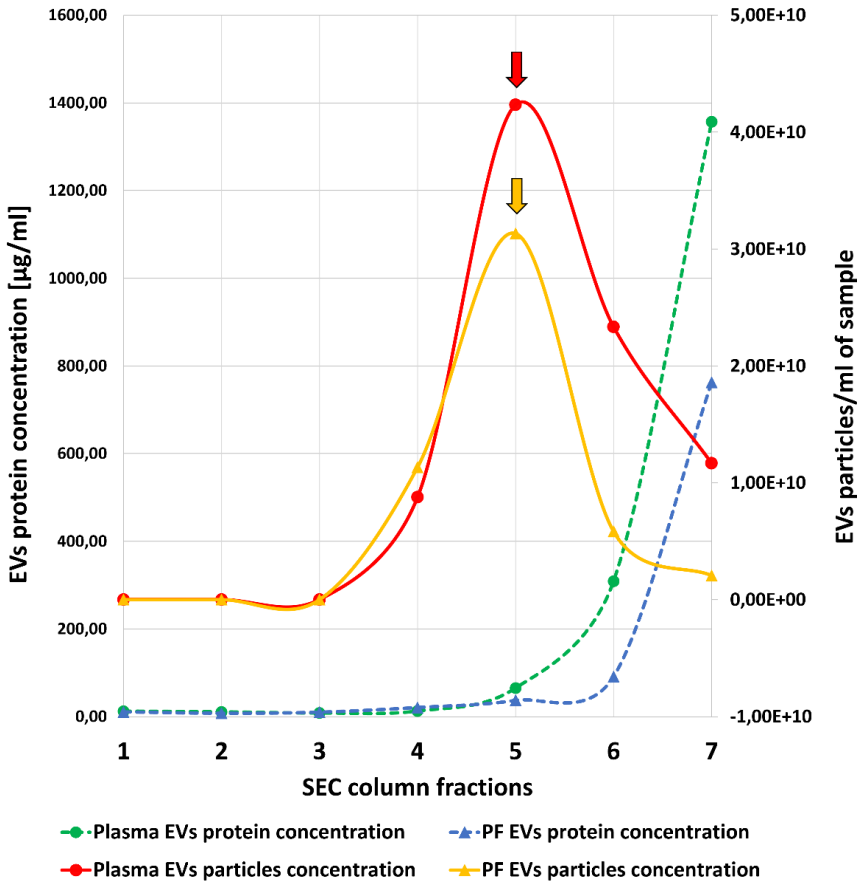

Figure 4. Distribution of EV particle and protein concentrations across SEC fractions obtained from plasma and PF. Seven sequential fractions were collected from 1 mL of plasma or PF using SEC. EV particle

concentration was quantified by NTA, and total protein concentration was measured using the BCA assay. Plasma-derived samples are presented as follows: EV particle concentration (red solid line) and protein concentration (green dashed line). PF-derived samples are shown as EV particle concentration (yellow solid line) and protein concentration (blue dashed line). Particle concentrations peaked in fraction 5 for both plasma and PF (indicated by arrows), identifying this fraction as EV-enriched. In contrast, total protein concentration increased predominantly in later fractions (6-7), consistent with the elution of soluble protein contaminants.

Particle concentration was determined by NTA, whereas total protein content was measured using the BCA assay. In both plasma and PF samples, particle concentration remained low in fractions 1-3 and increased markedly from fraction 4, reaching a maximum in fraction 5 (~4.3×10^{10} particles/mL for plasma and ~3.5×10^{10} particles/mL for PF), followed by a gradual decline in fractions 6 and 7. In contrast, protein concentration began to increase from fraction 6 and continued to rise in fraction 7 (~1357 µg/mL for plasma and ~762 µg/mL for PF), with further increases observed in subsequent fractions (not shown). Early fractions (1-3) contained negligible particle counts and low protein levels in both plasma and PF samples. Overall, plasma samples exhibited higher particle and protein concentrations than PF across corresponding fractions, consistent with the greater molecular complexity and protein content of circulating blood.

To further assess the purity of SEC fractions, the particle-to-protein ratio was calculated for each fraction obtained using self-prepared SEC columns used as an indicator of EV enrichment (Figure 5). Particle concentrations determined by NTA and protein content measured by BCA assay were used for this analysis. Fractions 4–5 exhibited the highest particle-to-protein ratios in both plasma and PF samples, indicating effective enrichment of EVs relative to soluble protein contaminants. Fractions exceeding 10^8 particles per µg protein were considered indicative of EV enrichment, consistent with previously proposed benchmarks for EV purity[128]. The dashed line represents the approximate threshold used to define EV-enriched fractions. Based on these results, fractions 4–5 (highlighted in grey) obtained from laboratory-prepared SEC columns were selected for EV characterization in accordance with MISEV guidelines[76] (including NTA, Western blotting, and cryo-EM).

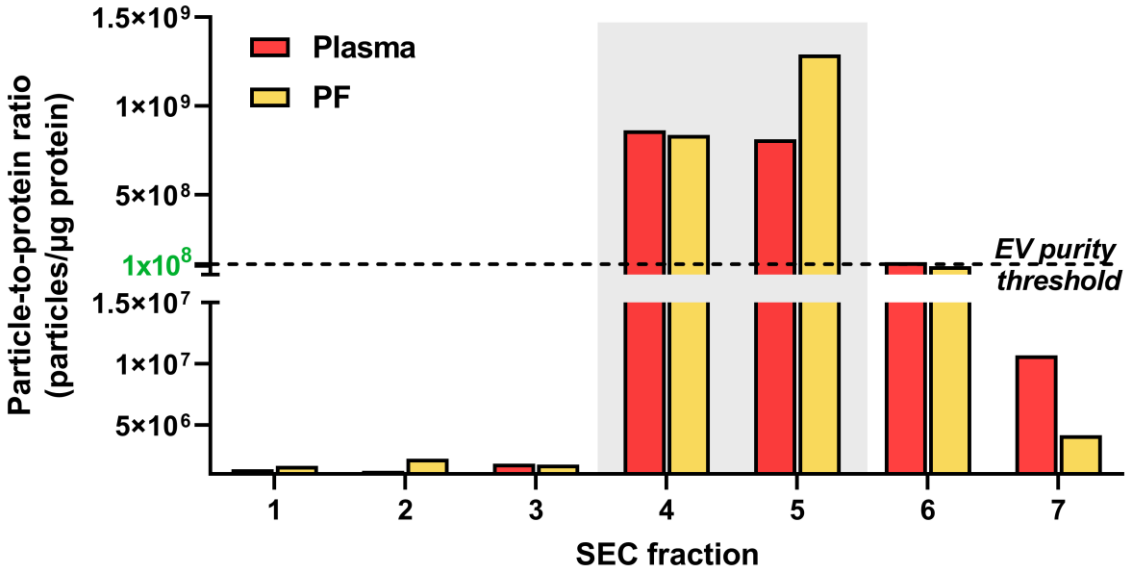

Figure 5. Particle-to-protein ratio across SEC fractions of plasma and PF samples. Particle-to-protein ratios calculated from NTA-derived particle concentrations and BCA protein measurements were used as an indicator of EV enrichment across SEC fractions. Fractions 4-5 exhibited the highest ratios in both plasma and PF samples, indicating enrichment of EVs relative to soluble protein contaminants. The dashed line represents the approximate threshold used to define EV-enriched fractions. Fractions 4-5 (highlighted in grey) were used for EV characterization in accordance with MISEV guidelines.

The observed elution profiles in both plasma and PF samples demonstrated effective separation of EV-enriched fractions from soluble protein contaminants, with fraction 5 showing the highest particle enrichment relative to protein content. These findings support the suitability of SEC

For proteomic analysis and IFCM measurements, EVs were isolated using commercially available qEV Original Gen 2 SEC columns (Izon Science). The elution profile obtained with these columns was consistent with the enrichment pattern observed using laboratory-prepared SEC columns, with EV-enriched fractions collected according to the manufacturer's protocol. The use of commercially available columns ensured higher reproducibility and standardization of EV isolation for downstream proteomic analyses.

Comprehensive characterization of EVs isolated from plasma and PF confirmed the effectiveness of the isolation procedure and their compliance with MISEV guidelines[76] (Figure 6). NTA analysis demonstrated that plasma-derived EVs ranged from 50 to 150 nm in size, whereas PF-derived EVs exhibited a broader size distribution of 50 to 400 nm (Figure 6A). Moreover, F-NTA confirmed the presence of lipid membrane-enclosed vesicles, as indicated by CMDR staining, and demonstrated the presence of EV-associated markers, including the tetraspanins CD9, CD81, and CD63 for PF EVs, and CD9 alone for plasma EVs (Figure 6B).

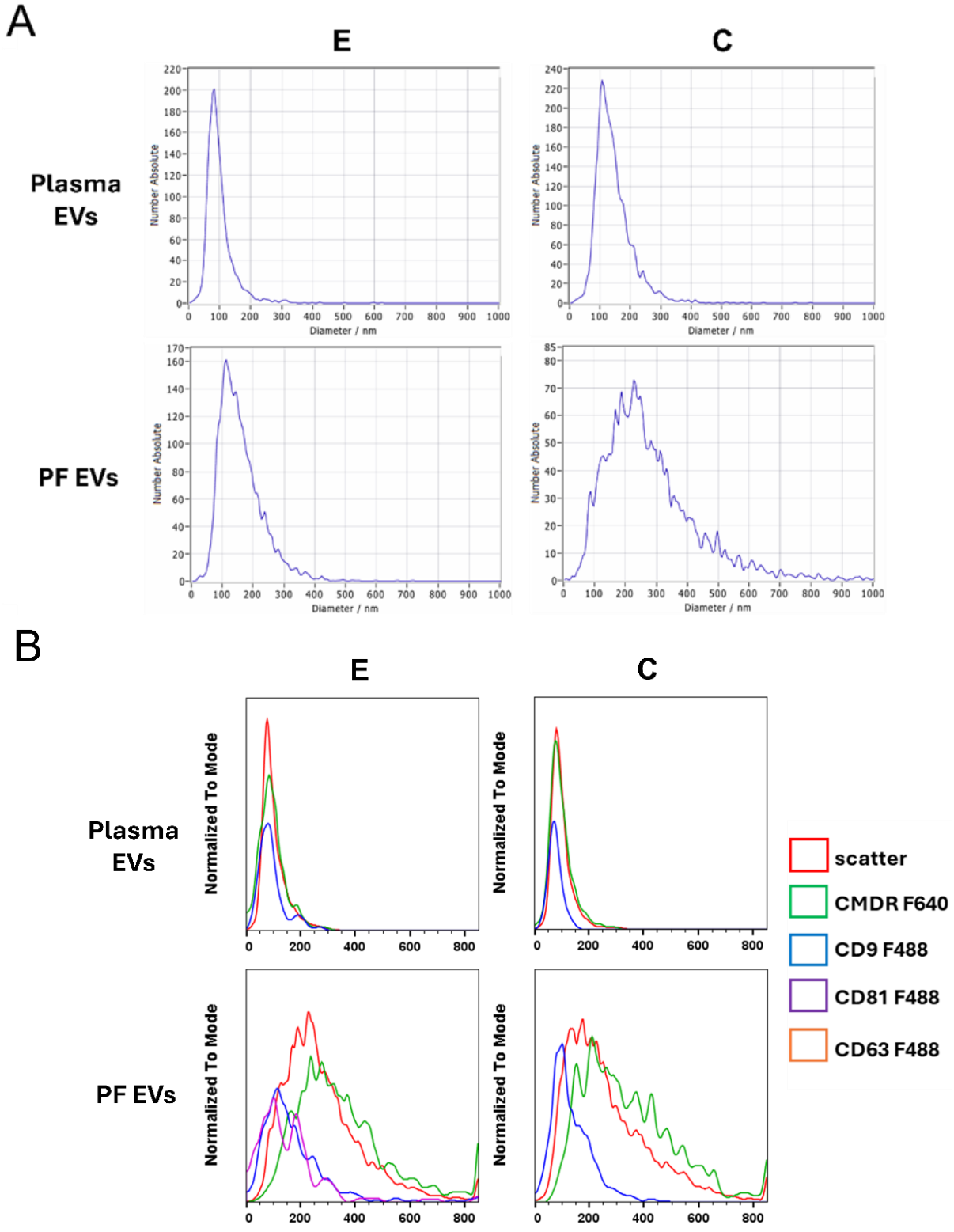

Figure 6. NTA-based assessment of size distribution and fluorescence profiles of EVs isolated from plasma and PF of patients with endometriosis (E) and controls (C). (A) Representative scatter mode NTA histograms showing particle size distribution and concentration of isolated vesicles from plasma and PF samples of E and C. (B) Representative F-NTA profiles demonstrating CMDR-positive vesicles indicating the presence of a lipid membrane, as well as tetraspanin-positive small EV subpopulations (CD9, CD81, CD63) in plasma and PF samples from E and C.

Additionally, cryo-EM imaging revealed single, round structures measuring 150-200 nm, exhibiting a distinct lipid bilayer with a thickness of approximately 4 nm, consistent with the characteristic morphology of small EVs (Figure 7).

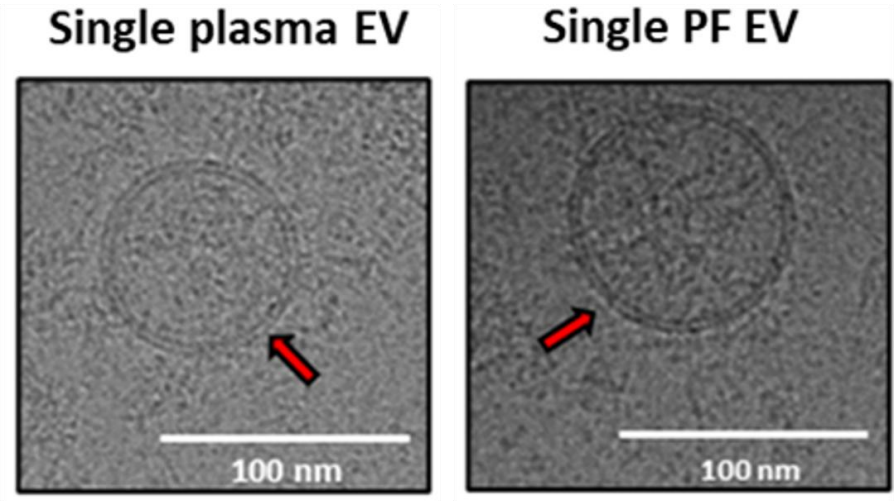

Figure 7. Cryo-EM imaging of plasma and PF EVs of an endometriosis patient. Representative cryo-EM images showing single EVs isolated from plasma (left) and PF (right). Red arrows indicate individual vesicles exhibiting a characteristic spherical morphology with a clearly visible lipid bilayer structure. Scale bar: 100 nm.

Western blotting analysis, presented in Figure 8, further confirms the presence of the aforementioned surface EV markers, such as CD9 and CD81, as well as additional intraluminal EV marker proteins, including EDH4 and Tsg101, in both plasma and PF EVs. Notably, the non-EV marker calnexin is detected only in the cell lysate, highlighting the efficiency of the EV separation process and the absence of ER contamination.

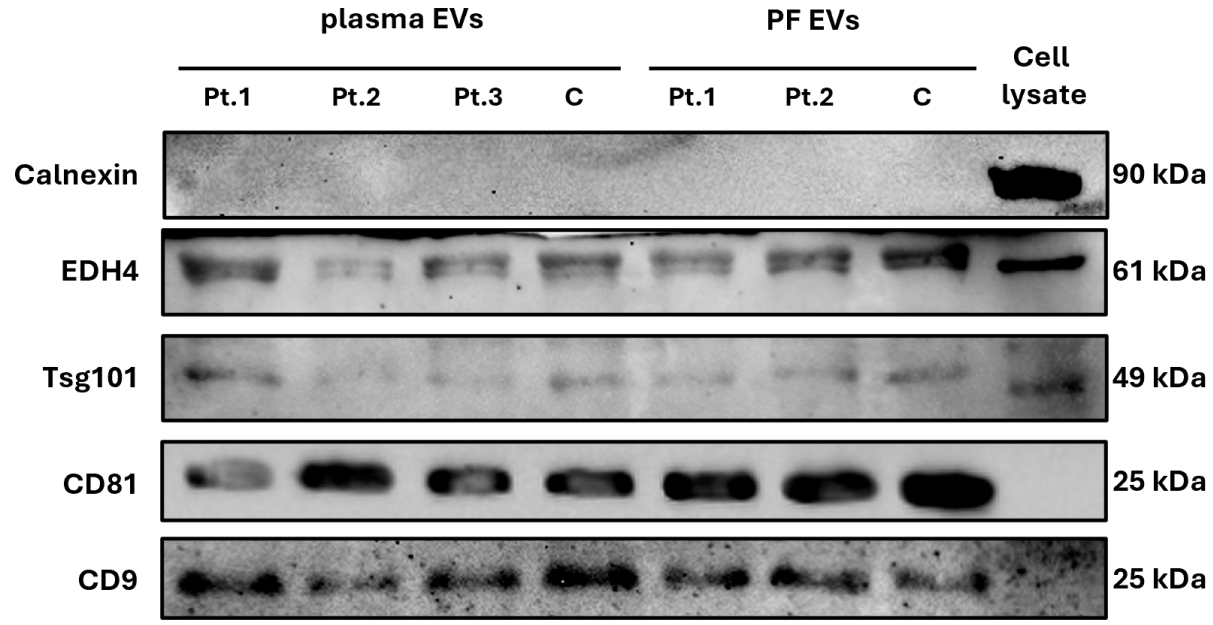

Figure 8.Western blotting analysis of EV-associated proteins in EVs isolated from plasma and PF of patients with endometriosis (E) and controls (C). Representative Western blot showing the presence of EV-

associated proteins EDH4, Tsg101, CD81, and CD9 in EV samples isolated from plasma and PF samples from E and C. Calnexin was used as a negative control for endoplasmic reticulum contamination.

Having confirmed successful isolation and molecular characterization of EVs, the next step was to assess whether quantitative differences in EV concentration and size could be detected between endometriosis patients and control subjects.

In plasma-derived EVs, scatter mode analysis revealed no significant difference in total particle concentration between groups (Figure 9A, left). In contrast, F-NTA restricted to CMDR-positive vesicles demonstrated a significantly higher concentration of membrane-labelled particles in the endometriosis group compared to controls (Figure 9A, right). Regarding particle size, scatter-based measurements showed a significant increase in mean EV diameter in plasma samples from endometriosis patients (Figure 9B, left). However, when the analysis was limited to CMDR-positive vesicles, no statistically significant size difference was observed between groups (Figure 9B, right).

In PF-derived EVs, no statistically significant differences were detected between endometriosis patients and controls in either particle concentration or size, in both scatter and fluorescence modes (data not shown). Together, these findings indicate that systemic (plasma) EV alterations in endometriosis are detectable at the level of membrane-positive vesicle abundance and scatter-defined size distribution, whereas no corresponding global quantitative differences were observed in PF-derived EVs.

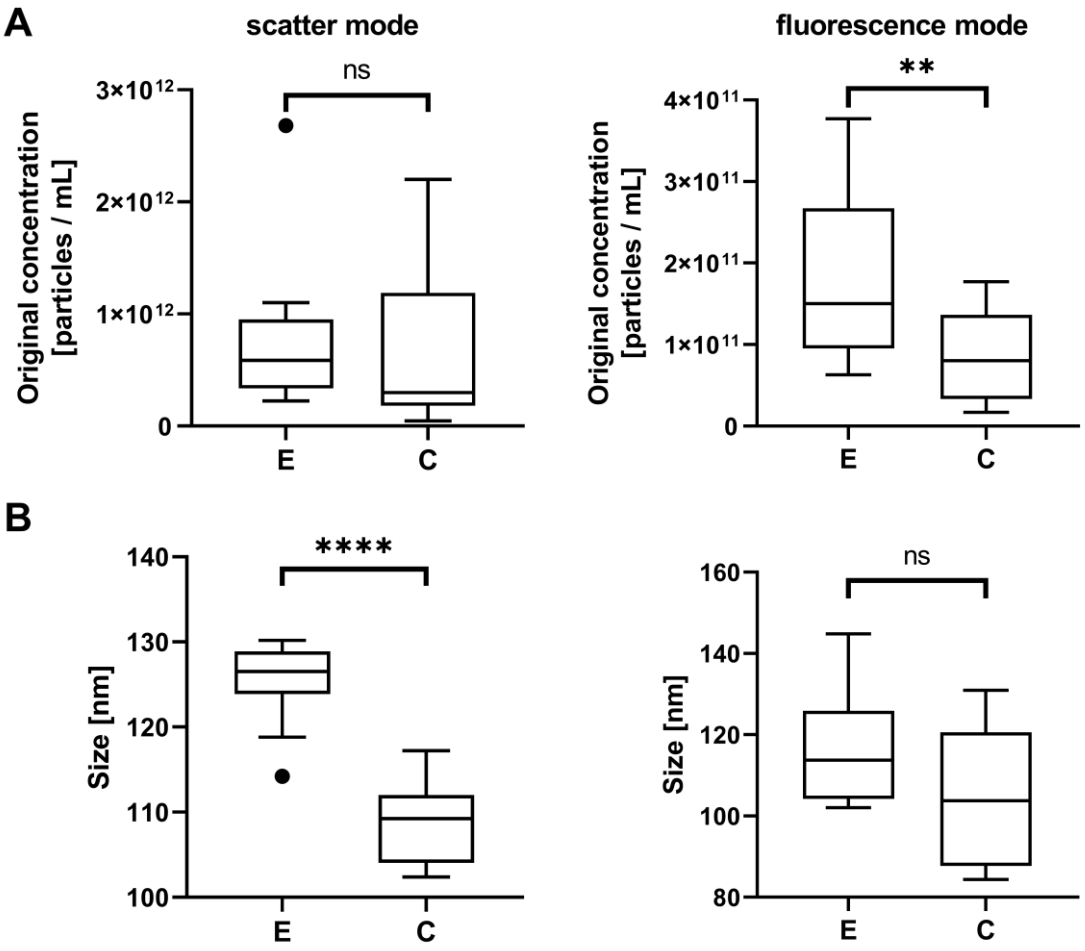

Figure 9. Quantitative analysis of plasma-derived EV concentration and size in endometriosis patients (E) and controls (C). (A) Original particle concentration (particles/mL) measured in scatter mode (left) and fluorescence mode using CellMask™ Deep Red (CMDR) (right). (B) Particle size (nm) determined in scatter mode (left) and fluorescence mode (right). Data are presented as boxplots showing the median and Tukey whiskers (1.5 × IQR). Statistical comparisons between groups were performed using the non-parametric Mann–Whitney U test. n = 15 per group. *p < 0.05, **p < 0.01, ****p < 0.0001; ns, not significant.

4.2. Distinct proteomic signatures in plasma and PF EVs in endometriosis

To investigate the proteomic cargo of EVs derived from plasma and PF, and to explore differences in their proteomic profiles between endometriosis and control patients as a basis for a future diagnostic and/or prognostic panel, high-throughput TMT-based proteomic analysis was performed. Given the systemic nature of plasma, in contrast to the close proximity of PF to endometrial lesions within the peritoneal cavity, EVs from both body fluids were analysed to provide a comprehensive understanding of the molecular alterations associated with endometriosis.

Global proteomic analysis identified and quantified 2,600 proteins in plasma-derived EVs and 3,571 in PF-derived EVs from endometriosis patients and controls. Among these, 2,304 proteins were common to both biofluids. By focusing exclusively on differentially

expressed proteins (DEPs) (p-value < 0.05) between endometriosis patients and controls, the dataset was reduced to 584 proteins showing either increased or decreased abundance in the endometriosis group relative to controls.

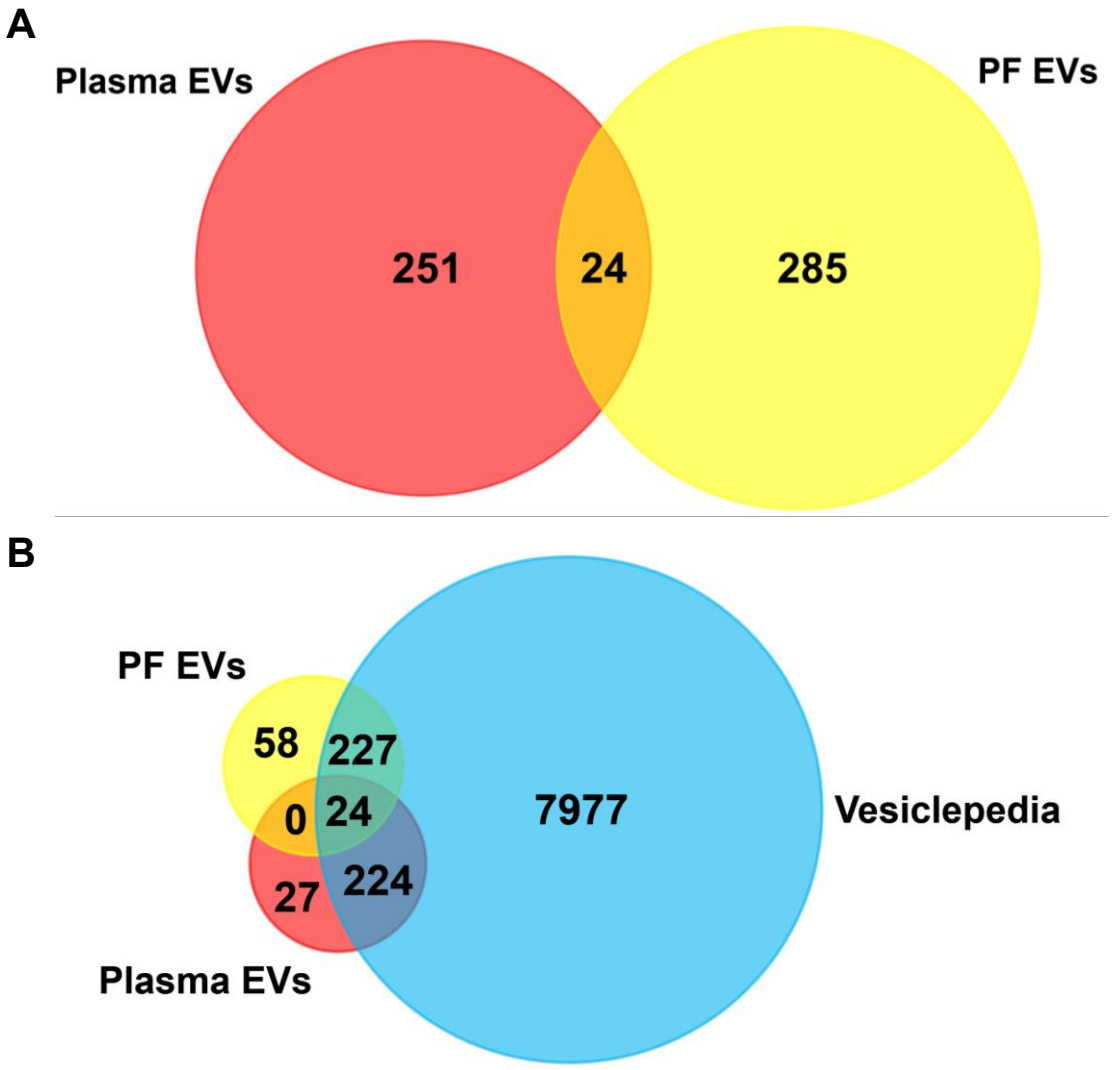

Figure 10. Overlap analysis of DEPs identified in EVs derived from plasma and PF of patients with endometriosis and their comparison with the Vesiclepedia database. (A) Venn diagram showing the number of DEPs (FDR < 0.01) identified in plasma EVs and PF EVs. A total of 251 DEPs were unique to plasma EVs, 285 were unique to PF EVs, and 24 DEPs were shared between both EV populations. (B) Venn diagram illustrating the overlap between proteins identified in plasma EVs, PF EVs, and proteins annotated in the Vesiclepedia database. The diagram shows the number of unique and shared proteins across the experimental datasets and the reference EV database.

Given their potential relevance to the disease, subsequent functional analyses were focused on this subset, comprising 275 proteins specific to plasma EVs, 309 unique to PF EVs, and 24 shared between both biofluids (Figure 10A). Furthermore, the majority of the identified EV proteins overlapped with entries in the Vesiclepedia database, a manually curated compendium of EV molecular cargo (including lipids, RNA, and proteins) derived

from multiple EV types[129], further supporting the reliability and biological relevance of these proteins (findings) in the context of EV-mediated mechanisms in endometriosis (Figure 10B).

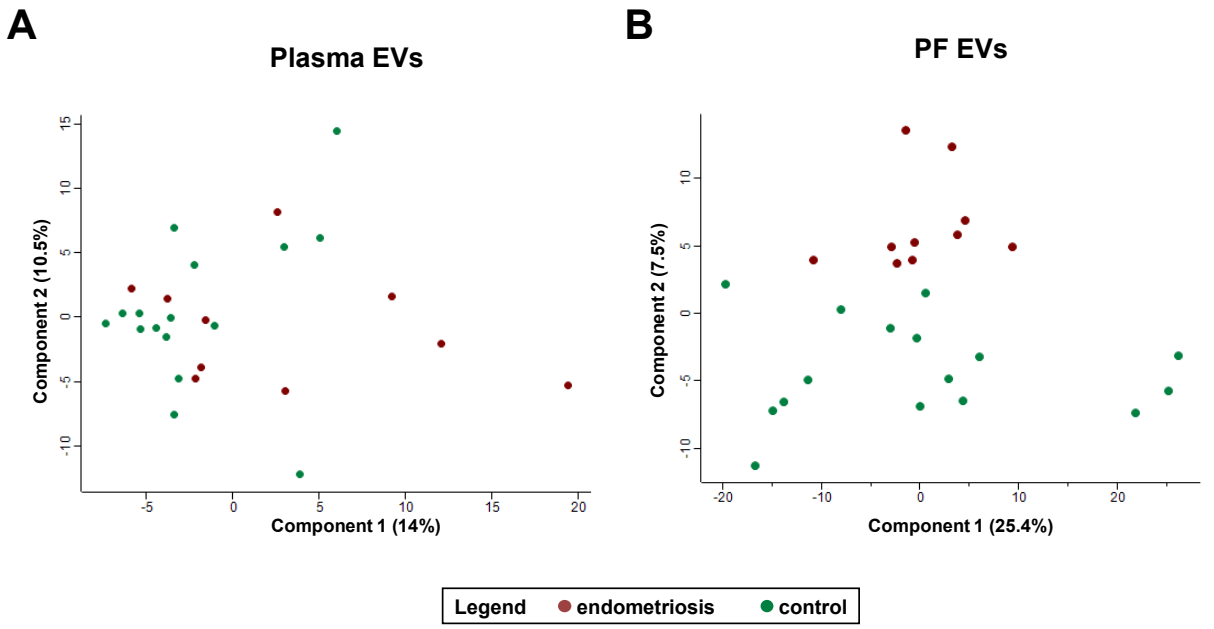

Figure 11. PCA of plasma and PF EV proteomes. PCA plots illustrating global variability in the proteomic profiles of plasma EVs (A) and PF EVs (B) in patients with endometriosis (red) and controls (green). Each point represents an individual sample (n = 10 patients with endometriosis and n = 16 controls). The analysis demonstrates partial separation between groups, indicating differences in EV proteomic composition.

The proteomic analysis revealed distinct molecular signatures in EVs from plasma and PF that differentiate endometriosis patients from controls. PCA (Figure 11) was used to assess variability in proteomic profiles between the two groups. For plasma-derived EVs (Figure 11A), the first two principal components (PC1 and PC2) accounted for 24.5% of the total variance (14% and 10.5%, respectively), showing partial separation between endometriosis and control samples, although the distinction between groups remained limited, suggesting moderate discriminatory power of plasma EV proteomic profiles.

In contrast, PF-derived EVs (Figure 11B) demonstrated stronger group discrimination. The PCA plot for PF EVs revealed tighter clustering of samples within each group and a clearer separation along the first two principal components, which together explained 32.9% of the total variance (PC1: 25.4%, PC2: 7.5%). These results indicate that PF-derived EVs capture more pronounced disease-associated molecular variation, enabling clearer differentiation between endometriosis and control groups.

4.3. Proteomic profiling and functional analysis of PF EVs

PF constitutes an essential microenvironment in direct proximity to regions actively implicated in endometriosis pathogenesis, including ectopic endometrial lesions. This anatomical closeness provides a unique opportunity to investigate disease-associated molecular alterations, making PF EVs particularly valuable for proteomic studies. Given their potential to provide a localized molecular snapshot of endometriosis pathophysiology, functional proteomic analysis of PF-derived EVs was prioritized to uncover key biological mechanisms underlying the disease.

The bar plot (Figure 12) presents the top 67 DEPs versus controls, with upregulated proteins highlighted in red and downregulated proteins in blue. Several proteins associated with immune signalling, ECM dynamics, and oxidative stress regulation displayed marked changes, suggesting their potential involvement in disease progression and peritoneal microenvironment remodelling. Some of the identified proteins showed very intense overexpression, including the top protein Hypermethylated in Cancer 2 (HIC2) with an outstanding 16.67-fold increase. HIC2 is a transcriptional repressor involved in development and cell differentiation, with a yet unknown role in endometriosis.

Among the strongly upregulated proteins were M-phase phosphoprotein 10 (MPHOSPH10), involved in ribosome biogenesis and pre-rRNA processing during the M phase of the cell cycle; mucin 5AC (MUC5AC), a secreted gel-forming mucin; FAT atypical cadherin 3 (FAT3), implicated in tissue morphogenesis and remodelling; and DNA methyltransferase 1 (DNMT1). To date, these proteins have not been directly linked to endometriosis. Downregulated proteins (ranging from 1.22- to 2.19-fold decrease) were less abundant and included, among others, SRA stem–loop interacting RNA binding protein (SLIRP), involved in mitochondrial RNA stability and post-transcriptional regulation, and pelota homolog (PELO), which participates in ribosome rescue during stalled translation, ensuring proper protein synthesis fidelity. In these cases as well, their role in endometriosis remains unclear.

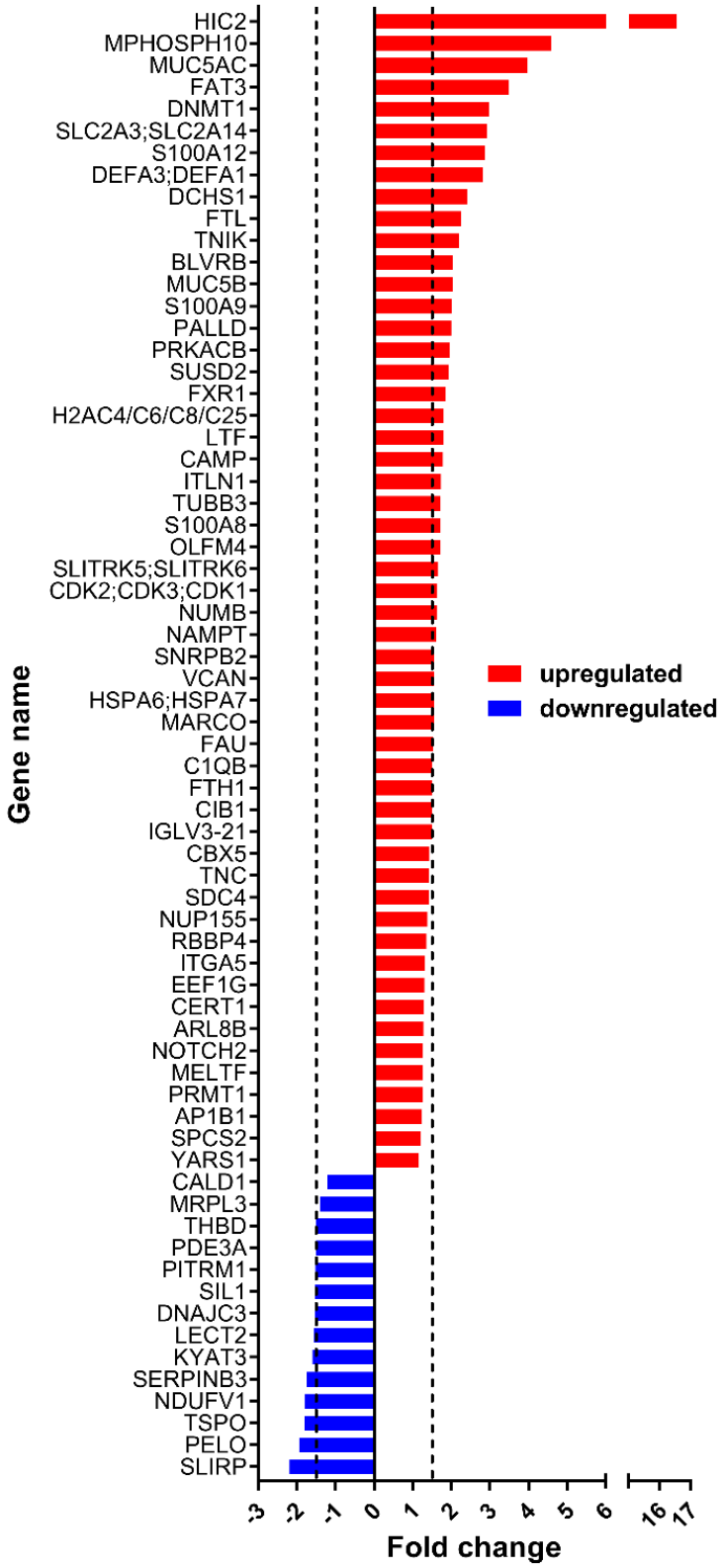

Figure 12. Differential proteomic signature of PF EVs in endometriosis. Bar plot presenting the most significantly DEPs identified in PF EVs from endometriosis patients compared with controls, ranked according to signed FC. Upregulated proteins are shown in red (FC ≥ 1.5), whereas downregulated proteins are shown in blue (FC ≤ −1.5). Vertical dashed lines indicate the predefined signed FC threshold of ±1.5 applied for protein selection. Unsupervised hierarchical clustering (Figure 13) revealed a clear separation of

samples corresponding to endometriosis and control groups, indicating that PF EV proteomic signatures reflect disease-associated molecular alterations.

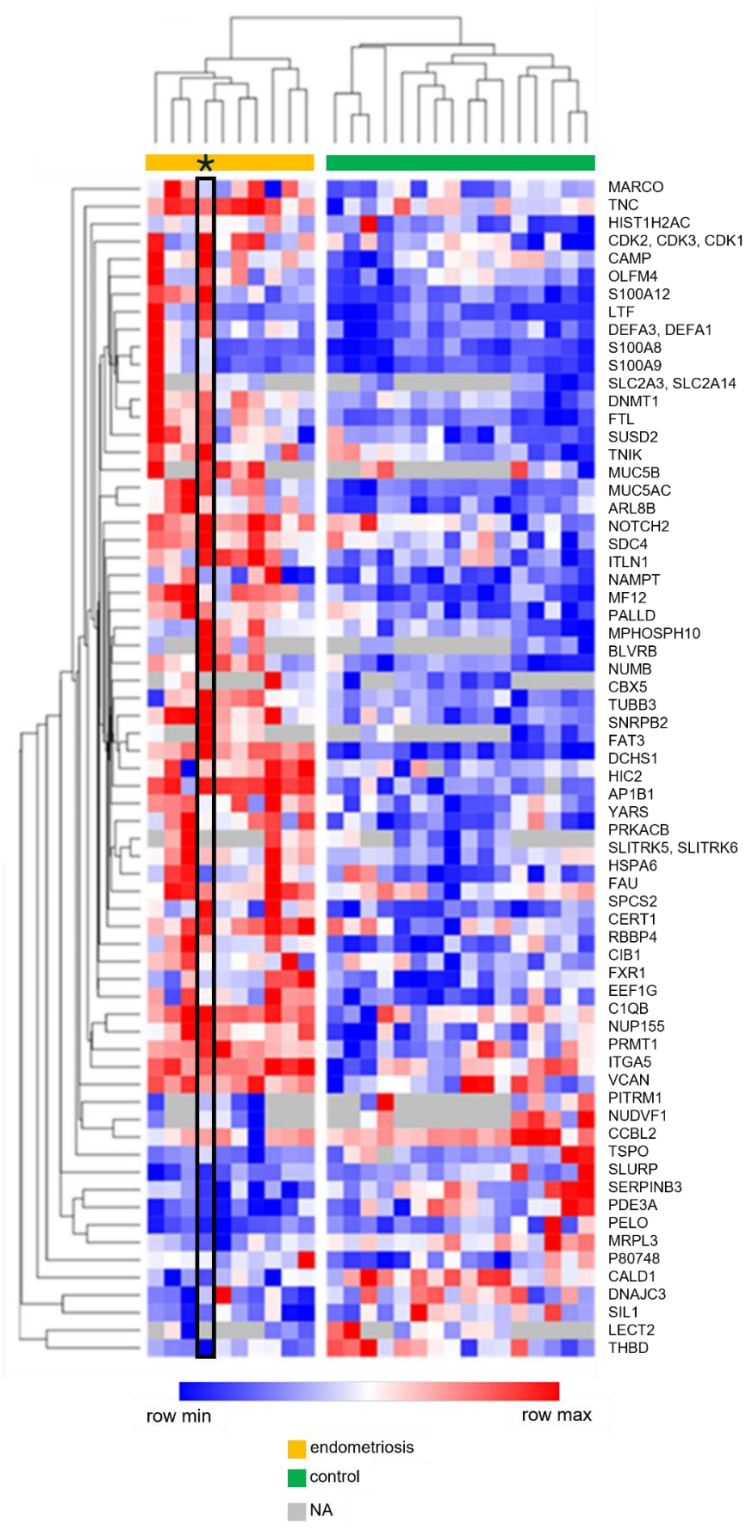

Figure 13. Hierarchical clustering heatmap of DEPs in PF EVs. Heatmap illustrating the relative abundance of DEPs in PF-derived EVs from patients with endometriosis and controls. Protein abundance values were standardized using row-wise Z-score normalization prior to clustering. Unsupervised hierarchical clustering was performed using Euclidean distance and average linkage. Colour intensity represents relative protein expression across samples (red - higher than the row mean; blue - lower than the row mean). Sample annotation bars indicate endometriosis (yellow), control (green), and missing values (NA, grey). The asterisk and black box

highlight a single patient diagnosed with stage IV endometriosis (rASRM stage IV), whereas the remaining patients were classified as stage III disease (rASRM stage III).

Notably, the expression profile of one patient clearly stood out, characterized by a high number of strongly upregulated proteins (indicated by an asterisk and a black box in the heatmap). Clinical data revealed that this sample corresponded to the only patient diagnosed with advanced stage IV endometriosis, whereas the remaining patients in the endometriosis group were classified as stage III disease. This observation suggests that EV proteomic profiles may reflect differences in disease severity. Taken together, these distinct clustering patterns support the potential of PF EV proteins as biomarkers reflecting localized molecular alterations associated with endometriosis.

A volcano plot (Figure 14) further visualized the distribution of DEPs, highlighting those with significant fold change (FC) values, thereby supporting their functional relevance in disease pathology.

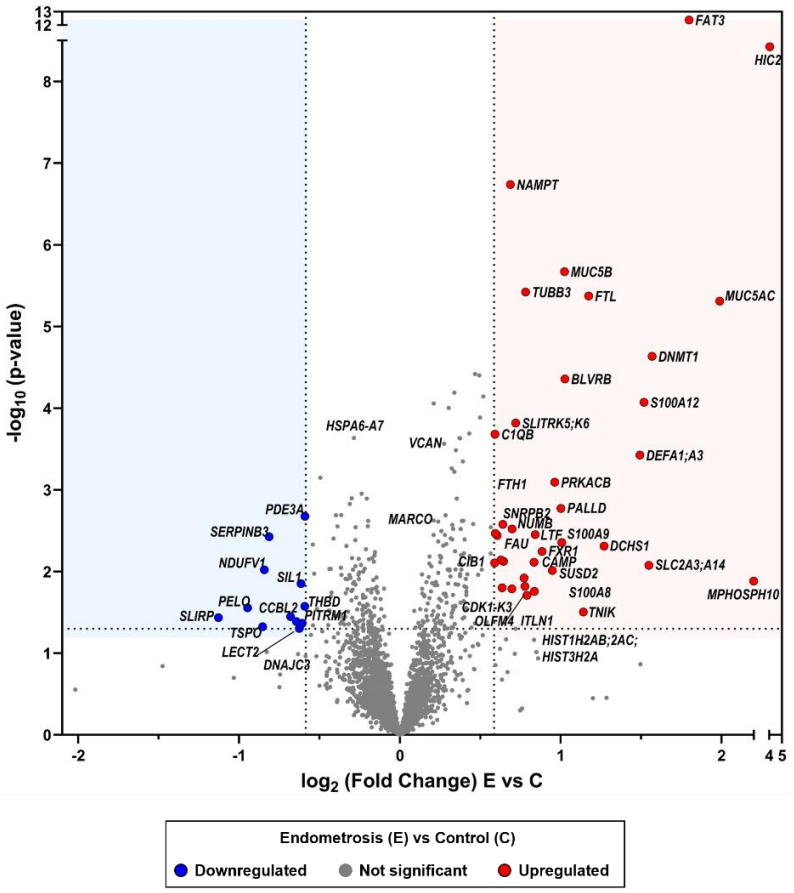

Figure 14. Volcano plot analysis of DEPs in PF EVs. Volcano plot illustrating the results of Student's t-test-based statistical analysis comparing PF-derived EVs from patients with endometriosis (E) and controls (C). The x-axis represents $\log_2$FC (E vs C), and the y-axis shows −log10(p-value) for each identified protein. Proteins meeting the predefined thresholds ($p < 0.05$ and $|\log_2 FC| \geq 0.585$) are highlighted in red (upregulated in E) and blue (downregulated in E), whereas non-significant proteins are shown in grey. Vertical dashed lines indicate the

log_2FC cutoffs corresponding to a FC threshold of ±1.5, and the horizontal dashed line represents the statistical significance threshold (p = 0.05).

To gain insights into the biological functions of these proteins, GO enrichment analysis was performed, identifying key pathways within the Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) (Figure 15). BP analysis (Figure 15A) revealed enrichment in pathways associated with innate immune response, neutrophil aggregation, and iron ion transport, suggesting that PF EVs may play an active role in modulating inflammatory and immune responses in endometriosis. MF enrichment analysis (Figure 15B) identified key molecular interactions, including receptor for advanced glycation end products (RAGE) binding and protein binding, implicating PF-derived EVs in immune receptor signalling. CC analysis (Figure 15C) in turn, confirmed that DEPs are strongly associated with extracellular compartments, including EVs and exosomes, supporting the role of PF EVs in intercellular communication within the peritoneal cavity.

Taken together, these findings suggest that PF EVs harbour a distinct proteomic signature enriched in immune-related, oxidative stress-associated, and ECM remodelling proteins, which may actively contribute to disease pathogenesis through immune dysregulation, inflammatory responses, and fibrosis. The distinct clustering of PF EV proteomes, along with their functional enrichment in key biological pathways, highlights their potential as diagnostic biomarkers and key modulators of the peritoneal microenvironment in endometriosis.

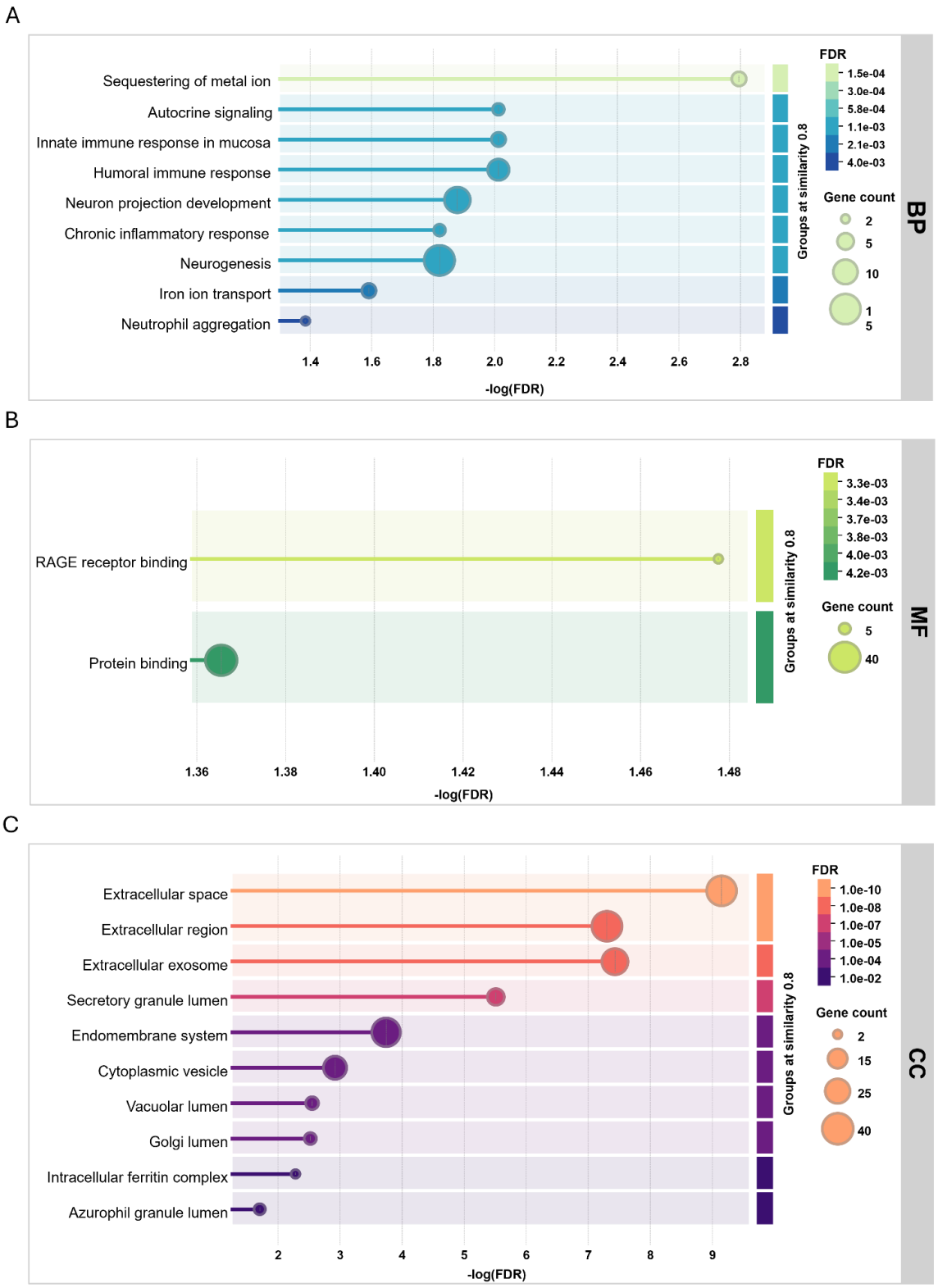

Figure 15. Functional enrichment analysis of DEPs in PF EVs from endometriosis patients. Gene GO enrichment analysis of DEPs across BP, MF, and CC. BP analysis reveals enrichment in immune-related processes, including innate immune response, neutrophil aggregation, and iron ion transport. MF terms highlight significant enrichment in RAGE receptor binding and protein binding. CC analysis indicates strong localization to extracellular compartments, including EVs and exosomes, supporting the role of PF EVs in intercellular communication

To gain further insight into the functional interactions among DEPs in PF EVs from endometriosis patients, a PPI network was constructed using the STRING database. The analysis revealed a significantly enriched interaction network (PPI enrichment p-value = 0.002), suggesting that these proteins are functionally interconnected and may play a role in endometriosis pathophysiology (Figure 16). The network was clustered into eight functional groups, highlighting key biological processes associated with endometriosis. Among these, proteins involved in metabolic adaptation and oxidative stress response (Cluster 1) and immune modulation and antimicrobial defence (Cluster 2) were highly interconnected, suggesting their potential role in the inflammatory microenvironment characteristic of the disease. Notably, proteins related to ECM interactions and adhesion (Cluster 5) and Notch signalling and cellular differentiation (Cluster 7) were also prominent, aligning with the well-established involvement of these pathways in ectopic tissue remodelling and lesion progression.

Several key proteins exhibited significant upregulation (red outer rings), including HIC2, MPHOSPH10, and MUC5AC, while downregulated proteins (blue outer rings), such as SLIRP, PELO, and TSPO, clustered within pathways related to mitochondrial function and cellular stress responses. The observed network topology underscores the functional relevance of PF EV-associated proteins in endometriosis and highlights potential molecular targets for further investigation.

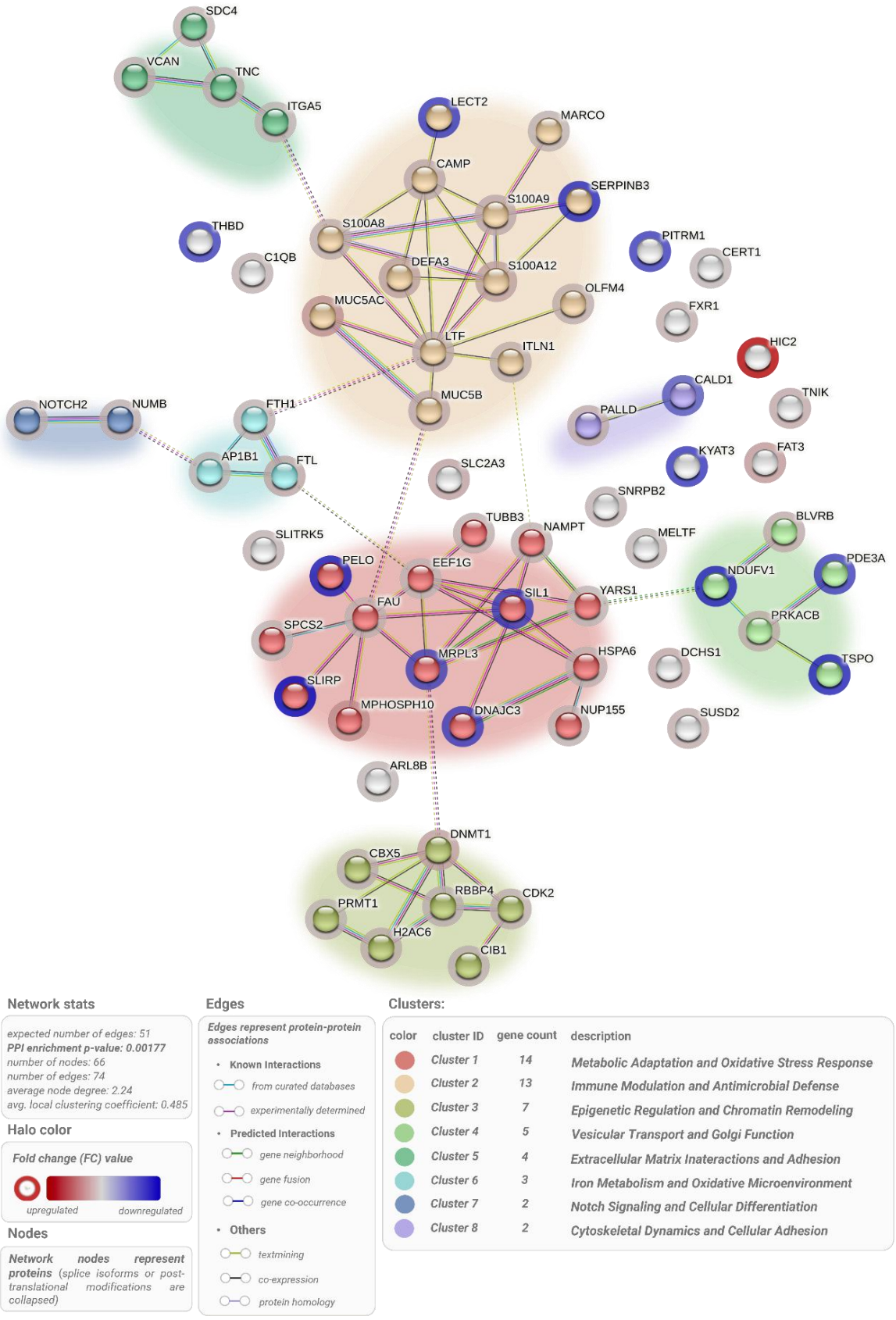

Figure 16. PPI network of DEPs in PF EVs from endometriosis patients. A PPI network was constructed using the STRING database, set to a medium confidence of 0.400 and prediction methods (i.e. genomic context prediction channels-neighbourhood, fusion, and co-occurrence; together with co-expression, text mining, curated databases, and experiments). 66 nodes (i.e. genes encoding functional proteins) were identified from an input of

67 genes at a PPI enrichment of p-value = 0. 002, indicating a significantly non-random interaction network. Upregulated (red) and downregulated (blue) proteins are highlighted, with edges representing known and predicted protein-protein associations. Nodes with at least 2 interactions are shown. PPI clusters were identified using the Markov Cluster Algorithm (MCL) with an inflation parameter of 1.3, optimizing the detection of tightly interconnected protein groups. Dashed lines indicate inter-cluster edges, representing interactions between distinct functional modules. Proteins are divided into 8 clusters, with functional annotations assigned based on their known biological roles. These include metabolic adaptation, immune modulation, ECM interactions, and Notch signalling, as determined by manual curation.

Reactome pathway enrichment analysis (Figure 17) was performed to explore the interconnecting functional roles of DEPs in PF EVs from endometriosis patients. The analysis identified significant enrichment of immune-related and ECM-associated pathways, highlighting the potential involvement of PF EV proteins in inflammatory responses and tissue remodelling characteristic of endometriosis. The bubble plot (Figure 17A) presents the most significantly enriched Reactome pathways, ranked by gene ratio (proportion of DEPs within each pathway). The innate immune system (HSA-168249) and neutrophil degranulation (HSA-6798695) pathways showed the highest enrichment, suggesting an active role of PF EV cargo in immune activation. Other significantly enriched pathways included antimicrobial peptides (HSA-6803157), metal sequestration by antimicrobial proteins (HSA-6799990), and scavenging by class A receptors (HSA-3000480), indicating the involvement of EV-associated proteins in host defence mechanisms. Bubble size reflects the number of proteins mapped to each pathway, while colour intensity represents statistical significance ($-\log_{10}$(p-value)), with red indicating the most significant enrichment.

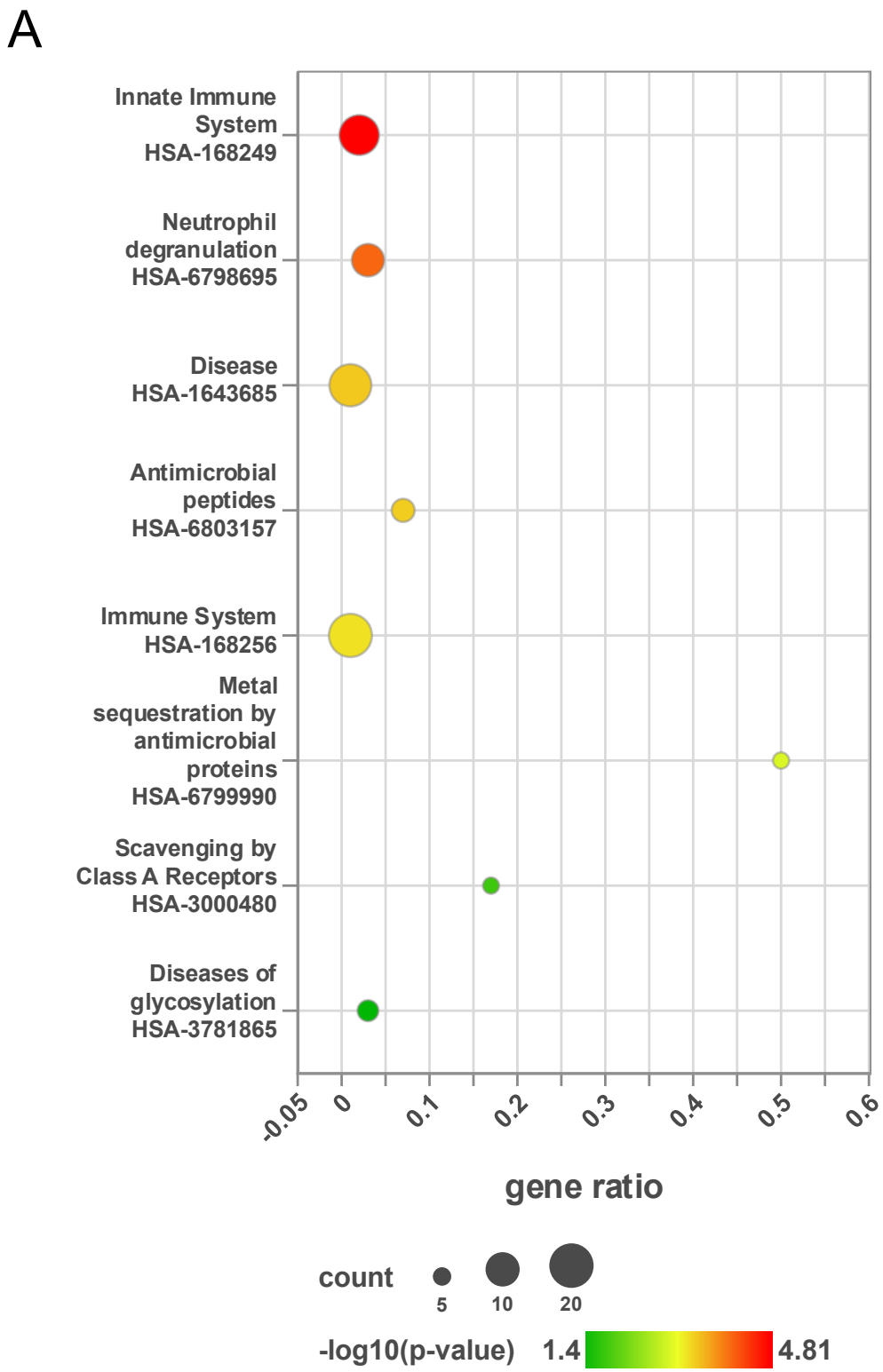

Figure 17. Reactome pathway enrichment analysis of DEPs in PF EVs from endometriosis patients. (A) Bubble plot showing significantly enriched Reactome pathways. The x-axis represents the gene ratio (proportion of DEPs within each pathway), while the y-axis lists the enriched pathways. The size of each bubble corresponds to the number of identified proteins within a given pathway, and the colour intensity reflects statistical significance, expressed as $-\log_{10}$(p-value), with red indicating the most significant enrichment.

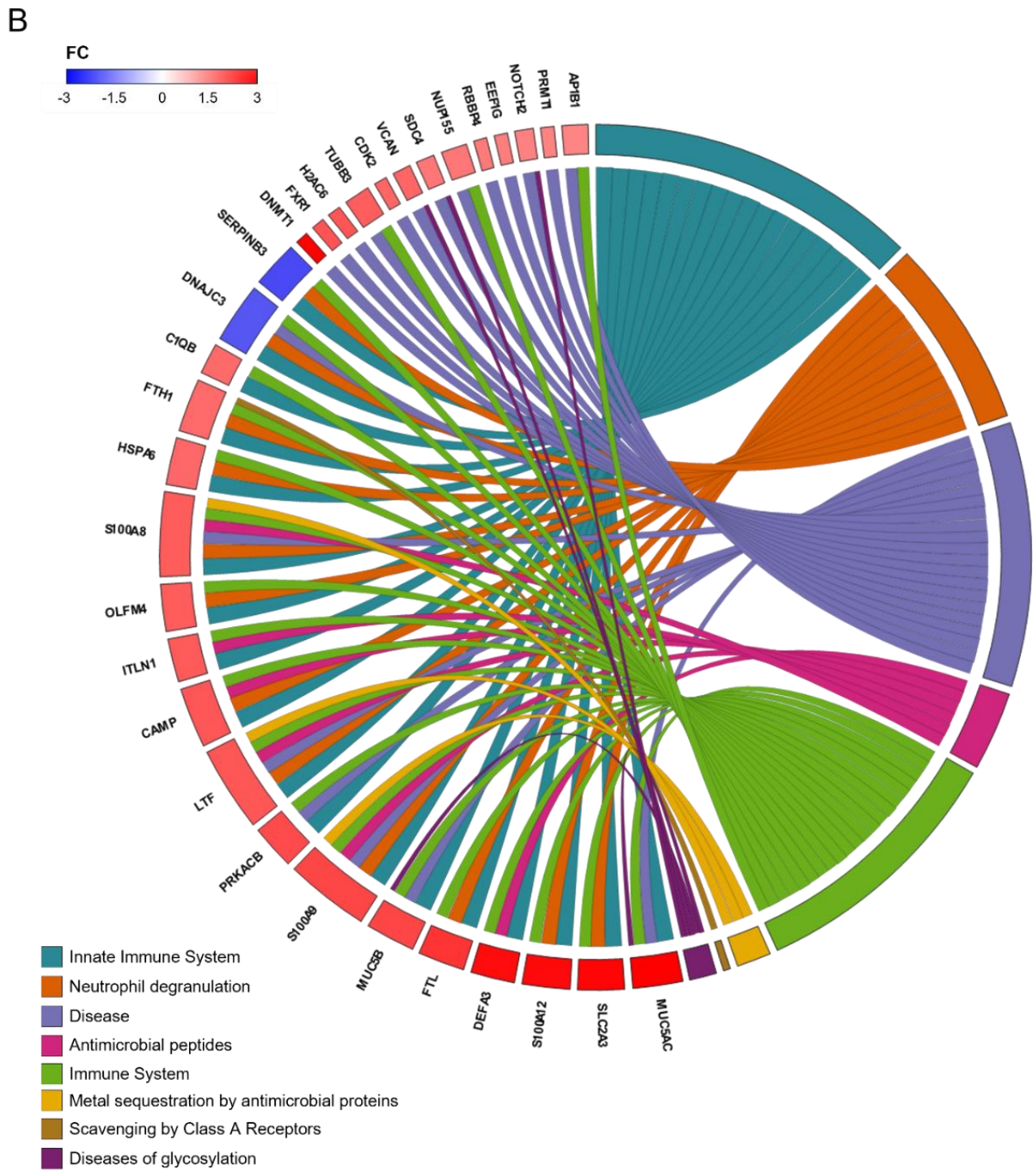

Figure 17. Continued. (B) Chord diagram illustrating the functional relationships between DEPs and enriched Reactome pathways. Pathways are colour-coded (right side of the diagram), and connecting lines represent associations between proteins (outer ring) and their corresponding biological processes, including Innate Immune System, Neutrophil Degranulation, Disease, Antimicrobial Peptides, Immune System, Metal Sequestration by Antimicrobial Proteins, Scavenging by Class A Receptors, and Diseases of Glycosylation. Upregulated proteins are shown in red, and downregulated proteins in blue, with FC values represented by a colour gradient (left side of the diagram).

To further investigate the functional connections between DEPs and enriched pathways, a chord diagram was generated (Figure 17B). The analysis revealed highly interconnected functional modules, with proteins mapped to innate immune system, neutrophil degranulation, and antimicrobial peptides pathways being predominantly upregulated, suggesting an enhanced immune response in the peritoneal microenvironment of endometriosis patients. The colour-coded edges represent functional interactions between proteins and pathways, with upregulated proteins in red, downregulated proteins in blue, and FC. The colour-coded edges represent functional interactions between proteins and pathways, with upregulated proteins in red, downregulated proteins in blue, and FC values depicted by a continuous colour gradient. These findings suggest that PF EV-associated proteins contribute to immune modulation and ECM remodelling in endometriosis, reinforcing their potential role as disease mediators or biomarkers.

Finally, KEGG pathway enrichment analysis was additionally performed to characterise pathway-level associations of PF EV-associated DEPs (Figures 18 and 19). The results revealed pathways associated with immune regulation, oxidative stress, and ECM remodelling, suggesting key roles for PF EV proteins in disease pathophysiology. The bubble plot (Figure 18) presents the most significantly enriched pathways, ranked by fold enrichment and statistical significance. Among them, necroptosis (hsa04217), IL-17 signalling (hsa04657), and systemic Lupus erythematosus (hsa05322) exhibited high enrichment, indicating an association between PF EV cargo and immune modulation. Additionally, pathways related to cell adhesion and ECM remodelling, such as ECM-Receptor interaction (hsa04512) and cell adhesion molecules (hsa04514), were significantly enriched, highlighting a potential role in tissue remodelling and fibrosis.

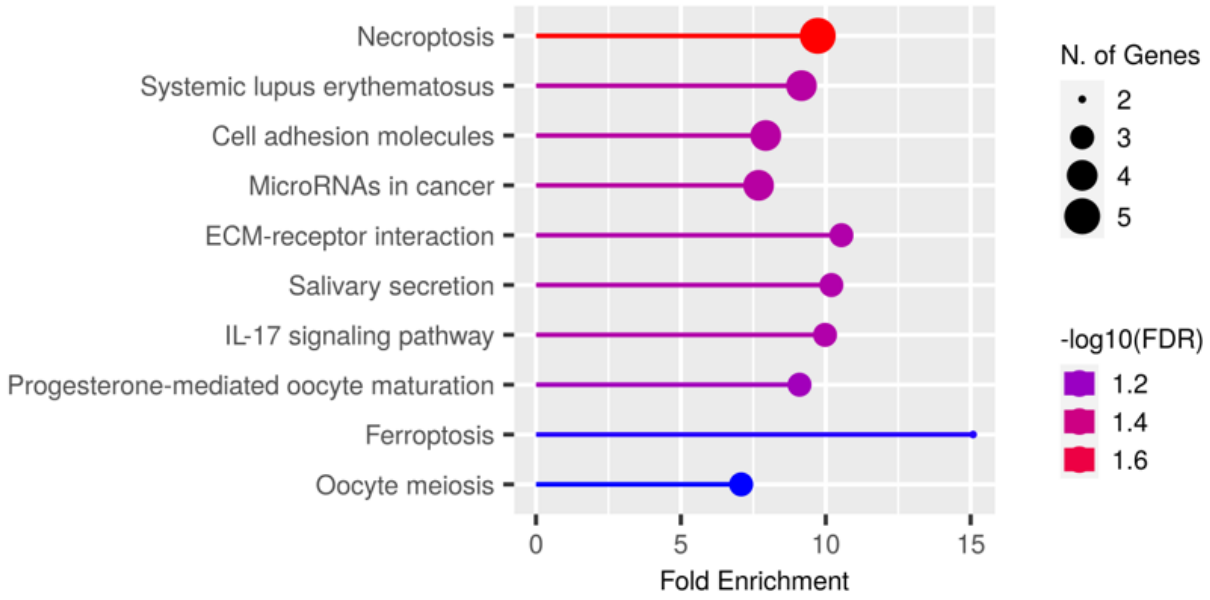

Figure 18. KEGG pathway enrichment analysis of DEPs in PF-derived EVs from endometriosis patients. Bubble plot showing significantly enriched KEGG pathways ranked by fold enrichment. Dot size represents the number of identified proteins, and colour intensity indicates statistical significance (-$\log_{10}$(FDR)).

To further explore these findings, DEPs were mapped onto selected KEGG pathways based on their enrichment strength and biological relevance to endometriosis, including IL-17 signalling, ferroptosis, and ECM–receptor interaction (Figure 19A–C). Only DEPs were highlighted in these pathway maps to emphasise proteins potentially contributing to disease-associated molecular alterations. Specifically, within the ferroptosis pathway (Figure 19A), transferrin receptor (TFRC) and solute carrier family 7 member 11 (SLC7A11) were upregulated, both involved in iron uptake and antioxidant response, while ferritin light chain (FTL), ferritin heavy chain 1 (FTH1), and glutathione peroxidase 4 (GPX4) were downregulated, indicating reduced iron storage and impaired oxidative stress protection, potentially increasing susceptibility to ferroptosis. In turn, within the ECM–receptor interaction pathway (Figure 19B), upregulation of osteopontin (OPN), thrombospondin 1 (THBS1), and collagen type VI alpha 3 chain (COL6A3), together with downregulation of integrin beta-1 (ITGB1) and integrin alpha-5 (ITGA5), points to excessive ECM remodelling and altered cell–matrix adhesion, favouring fibrosis and lesion progression. Finally, within the IL-17 signalling pathway (Figure 19C), mucin 5AC (MUC5AC), S100 calcium-binding protein A8 (S100A8), and S100 calcium-binding protein A9 (S100A9) were upregulated, while S100 calcium-binding protein A7 (S100A7) was downregulated, suggesting an enhanced pro-inflammatory response alongside altered epithelial immune defence.

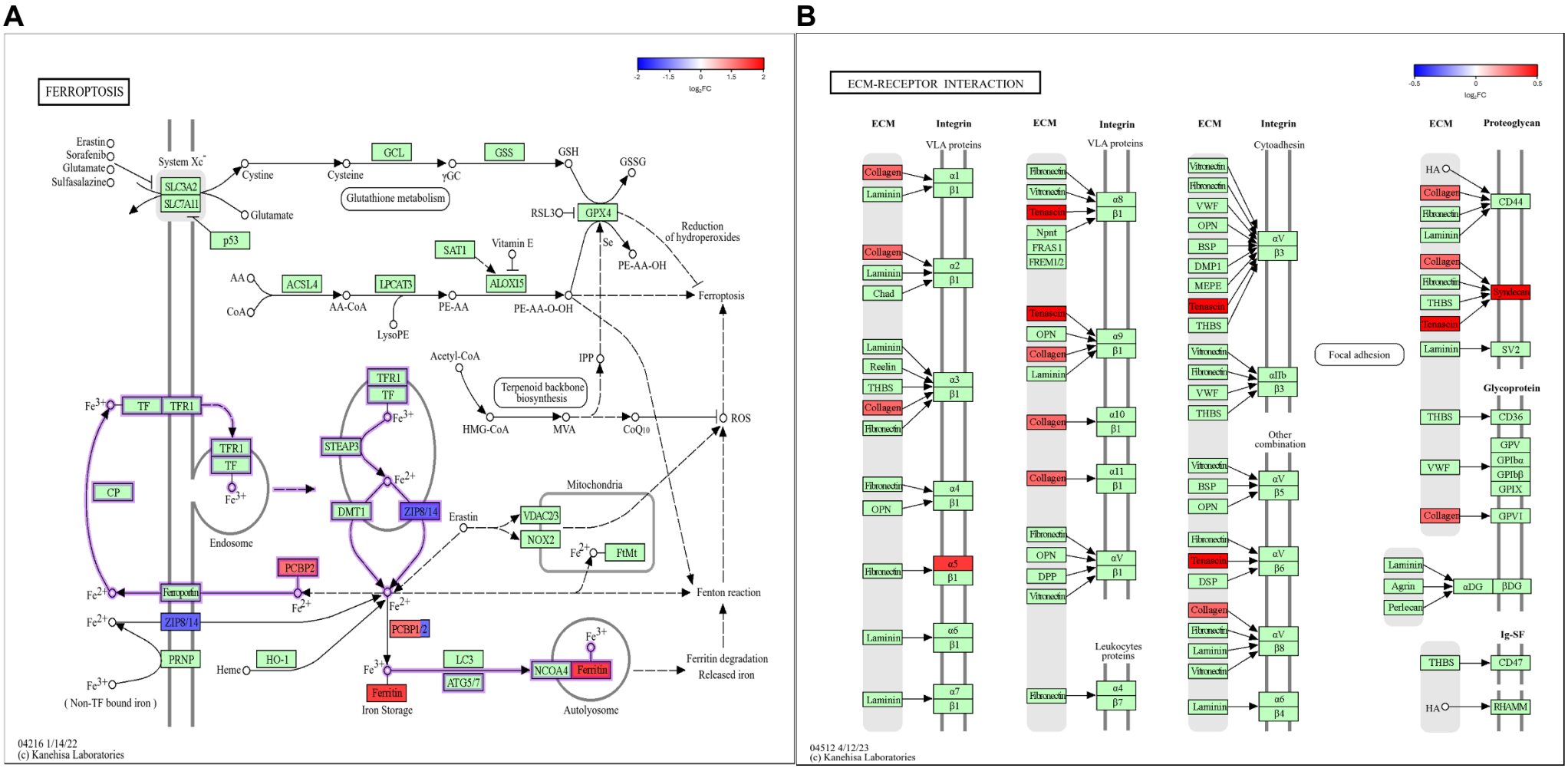

Figure 19. KEGG pathway enrichment and functional mapping of DEPs in PF-derived EVs from endometriosis patients. A) Bubble plot showing significantly enriched KEGG pathways ranked by fold enrichment. Dot size represents the number of identified proteins, while colour intensity indicates statistical significance (-$\log_{10}$(FDR). (B) Ferroptosis pathway illustrating EV-associated proteins involved in iron metabolism and lipid peroxidation. Upregulated proteins are shown in red, downregulated proteins in blue, with $\log_2$FC represented by colour intensity.

C

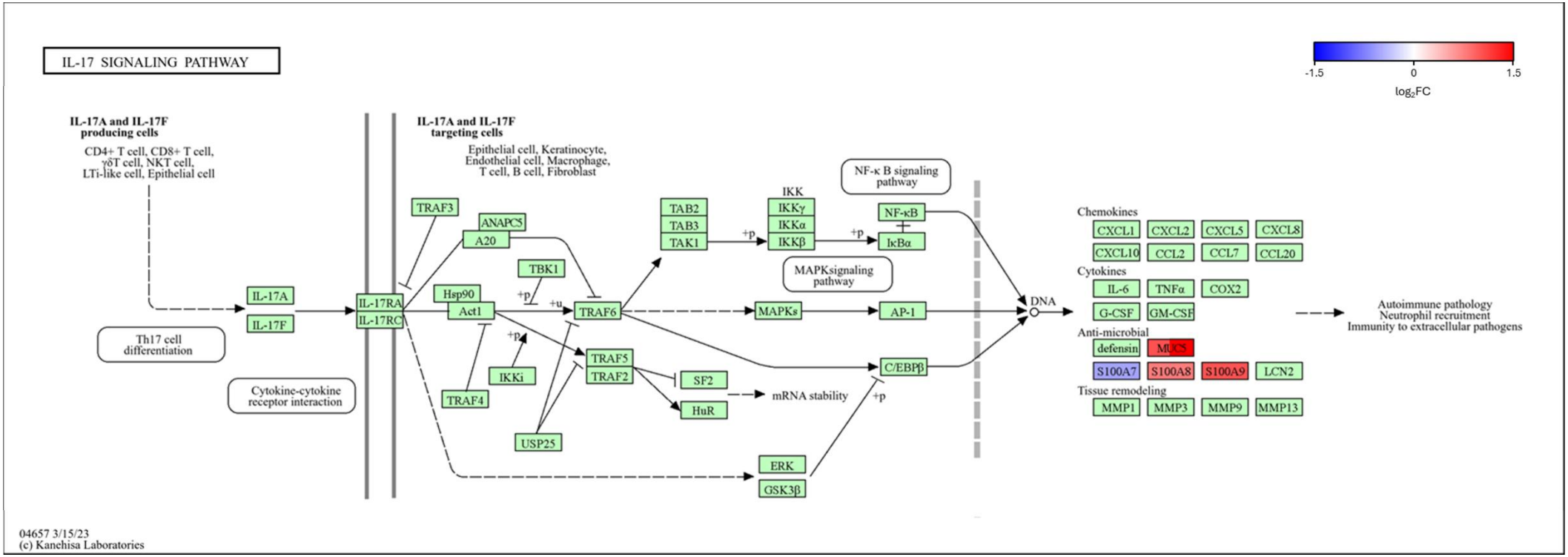

Figure 19. Continued. (C) ECM-receptor interaction pathway highlighting EV-associated proteins involved in ECM organisation and cell adhesion. Upregulated proteins are shown in red, downregulated proteins in blue, with log_2FC represented by colour intensity.

4.3. Proteomic profiling and functional analysis of plasma EVs

While PF-derived EVs provide a localized snapshot of the peritoneal microenvironment, plasma-derived EVs offer insight into the systemic manifestations of endometriosis. Plasma EVs, showed a more moderate discriminatory power compared to PF EVs, as observed in the PCA (Figure 11A) and hierarchical clustering (Figure 21). However, they still revealed a distinct set of DEPs that may reflect systemic immune responses, hormonal regulation, and metabolic alterations associated with the disease.

To explore the biological relevance of these proteomic differences in plasma-derived EVs, comprehensive functional analyses were conducted focusing on a subset of 36 differentially abundant proteins specific to plasma EVs. These proteins were subjected to GO enrichment analysis and PPI network mapping to uncover key molecular pathways and biological processes potentially involved in endometriosis pathology at the systemic level.

The bar plot (Figure 20) presents the top 36 DEPs in plasma-derived EVs from endometriosis patients compared to healthy controls, with upregulated proteins shown in red and downregulated proteins in blue. Several of the most altered proteins are implicated in vesicle trafficking, cell adhesion, and immune signalling, suggesting a role in systemic immune modulation and altered intercellular communication associated with endometriosis.

The top upregulated protein, with an outstanding 7.36-fold increase, was phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha (PIK3C2A), a member of class II PI3Ks implicated in the regulation of autophagy and endosomal sorting—processes thought to contribute to ectopic endometrial cell survival.

Several other strongly upregulated proteins (ranging from 1.17- to 7.37-fold increase) have previously been reported in association with endometriosis, including syndecan-4 (SDC4), a transmembrane heparan sulfate proteoglycan involved in cell adhesion, migration, inflammatory signalling, and angiogenesis, thereby promoting lesion establishment and maintenance. Mucin 16 (MUC16), also known as CA-125, has been investigated as part of biomarker panels for endometriosis diagnosis, although with limited sensitivity and specificity. Additionally, S100 calcium-binding protein A12 (S100A12), a member of the calcium-binding protein family, has been shown to modulate inflammatory pathways (e.g., NF-κB and RAGE signalling), enhancing lesion survival and inflammation.

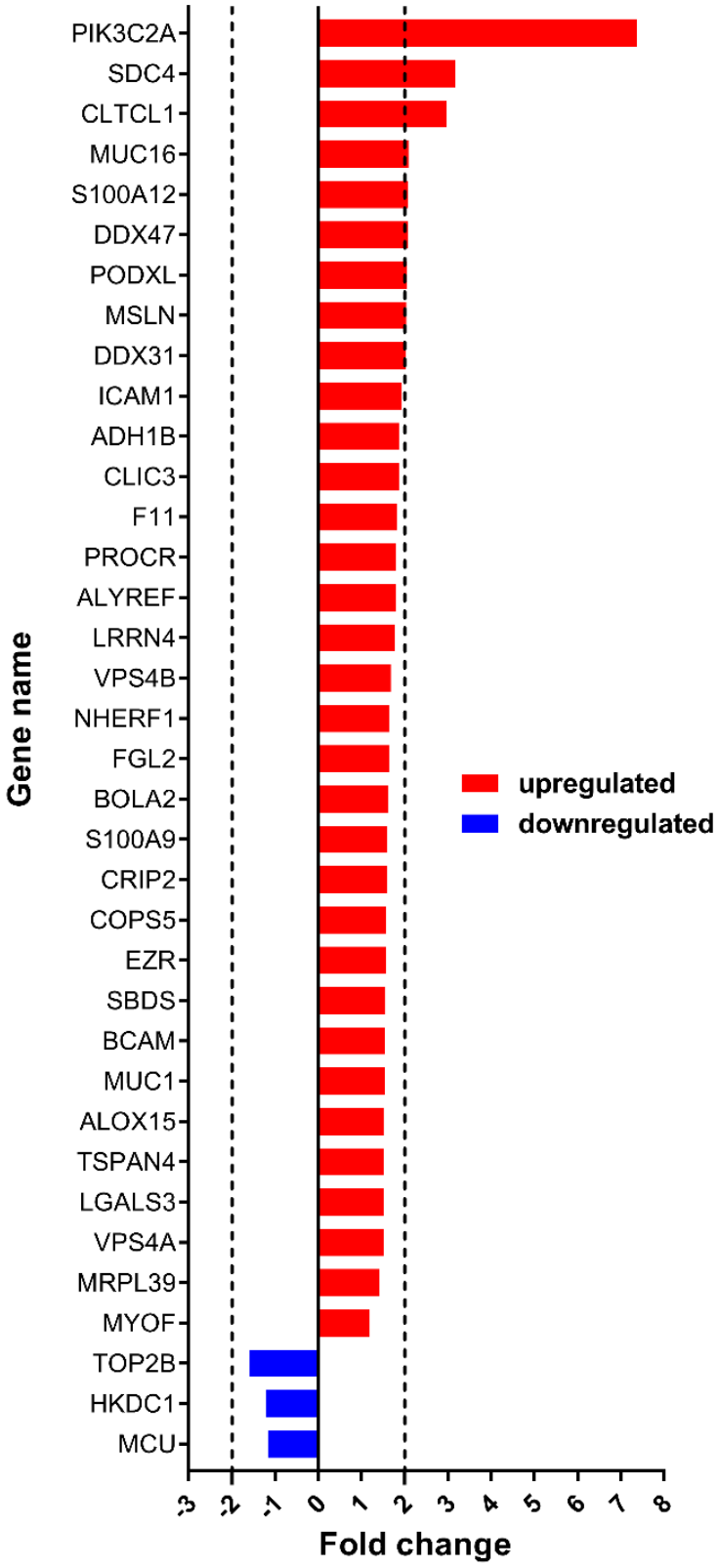

Figure 20. Differential proteomic signature of plasma EVs in endometriosis. Bar plot presenting the most significantly DEPs identified in plasma EVs from endometriosis patients compared with controls, ranked according to signed FC (values are presented as signed, where positive values indicate upregulation and negative values indicate downregulation). Upregulated proteins are shown in red (FC ≥ 1.5), whereas downregulated proteins are shown in blue (FC ≤ −1.5). Vertical dashed lines indicate the predefined FC threshold of ±1.5 applied for protein selection.

In contrast to the substantial number of upregulated proteins, only a few proteins with moderately reduced expression (ranging from 1.16- to 1.59-fold decrease) were identified, including DNA topoisomerase II beta (TOP2B), which is involved in the relaxation of supercoiled DNA during transcription and may contribute to alterations in chromatin remodelling and transcriptional regulation, as well as progesterone resistance—hallmarks of endometriotic cells.

In plasma EVs, clustering analysis also demonstrated separation between the study groups. Unsupervised hierarchical clustering (Figure 21) revealed a generally clear distinction between endometriosis patients and controls, although a certain degree of overlap was observed, with several control samples clustering close to those from endometriosis patients. This pattern suggests that, while plasma-derived EVs capture disease-related alterations, the proteomic differences are less pronounced and show greater variability compared with those observed in PF-derived EVs. Nevertheless, the overall clustering pattern supports the consistency of differential protein expression and highlights the potential of circulating EV-associated proteins as candidates for disease detection. Similarly to observations in PF-derived EVs, one patient with advanced endometriosis displayed a particularly distinct protein expression profile characterized by strong upregulation of multiple proteins (marked with an asterisk in the heatmap). This observation further supports the association between the identified protein expression patterns and disease progression, suggesting that the EV protein signature may also have potential utility for disease stratification.

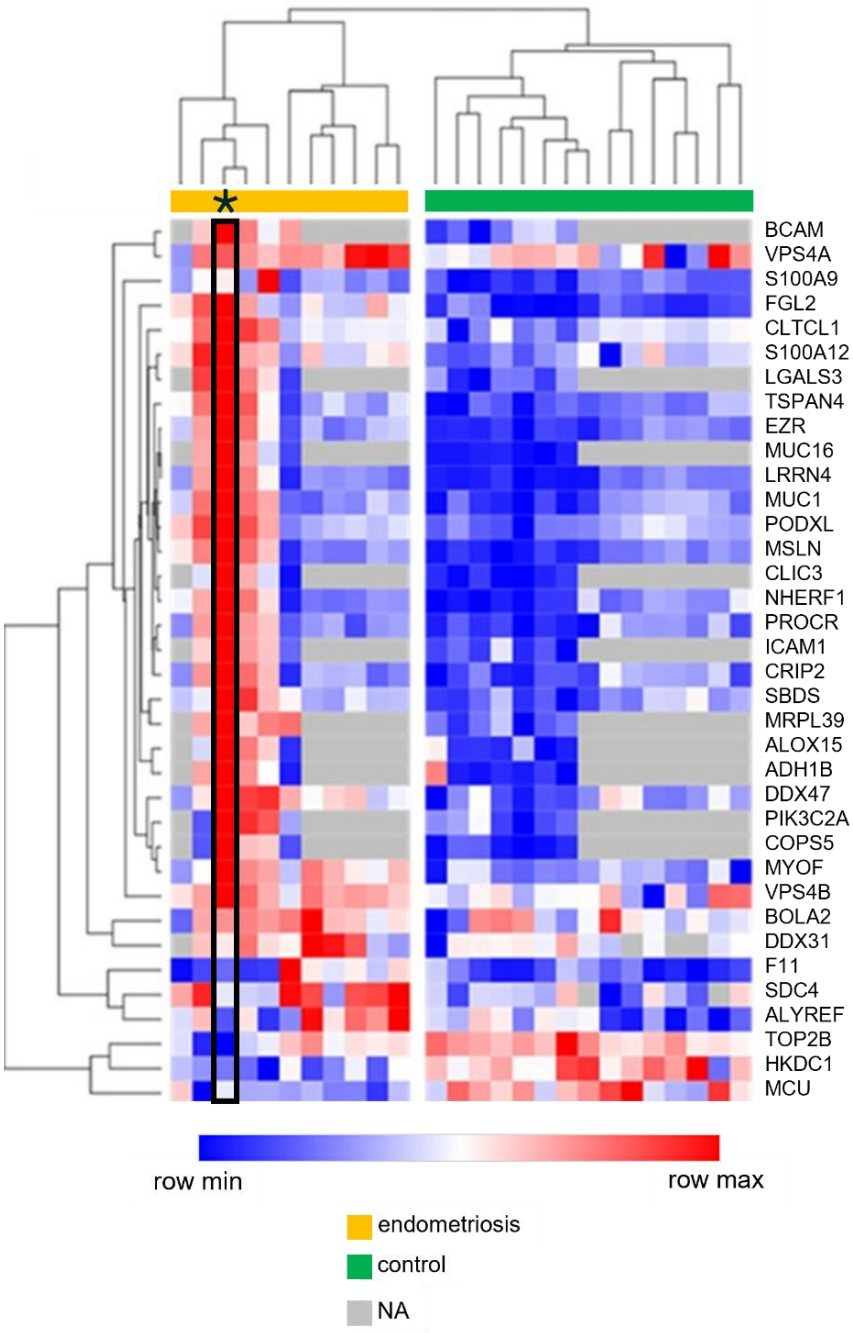

Figure 21. Hierarchical clustering heatmap of DEPs in plasma EVs. Heatmap illustrating the relative abundance of DEPs in plasma-derived EVs from patients with endometriosis and controls. Protein abundance values were standardized using row-wise Z-score normalization prior to clustering. Unsupervised hierarchical clustering was performed using Euclidean distance and average linkage. Colour intensity represents relative protein expression across samples (red - higher than the row mean; blue - lower than the row mean). Sample annotation bars indicate endometriosis (yellow), control (green), and missing values (NA, grey). The asterisk and black box highlight a single patient diagnosed with stage IV endometriosis (rASRM stage IV), whereas the remaining patients were classified as stage III disease (rASRM stage III).

The volcano plot (Figure 22) further illustrates the distribution of DEPs based on statistical significance and magnitude of change. Several proteins exhibited both high fold changes and low p-values, including PIK3C2A, SDC4, clathrin heavy chain-like 1 (CLTCL1), DEAD-box helicase 47 (DDX47), MUC16, S100A12, podocalyxin-like protein 1

(PODXL), intercellular adhesion molecule 1 (ICAM1), and TOP2B, highlighting their potential biological relevance in systemic manifestations of endometriosis.

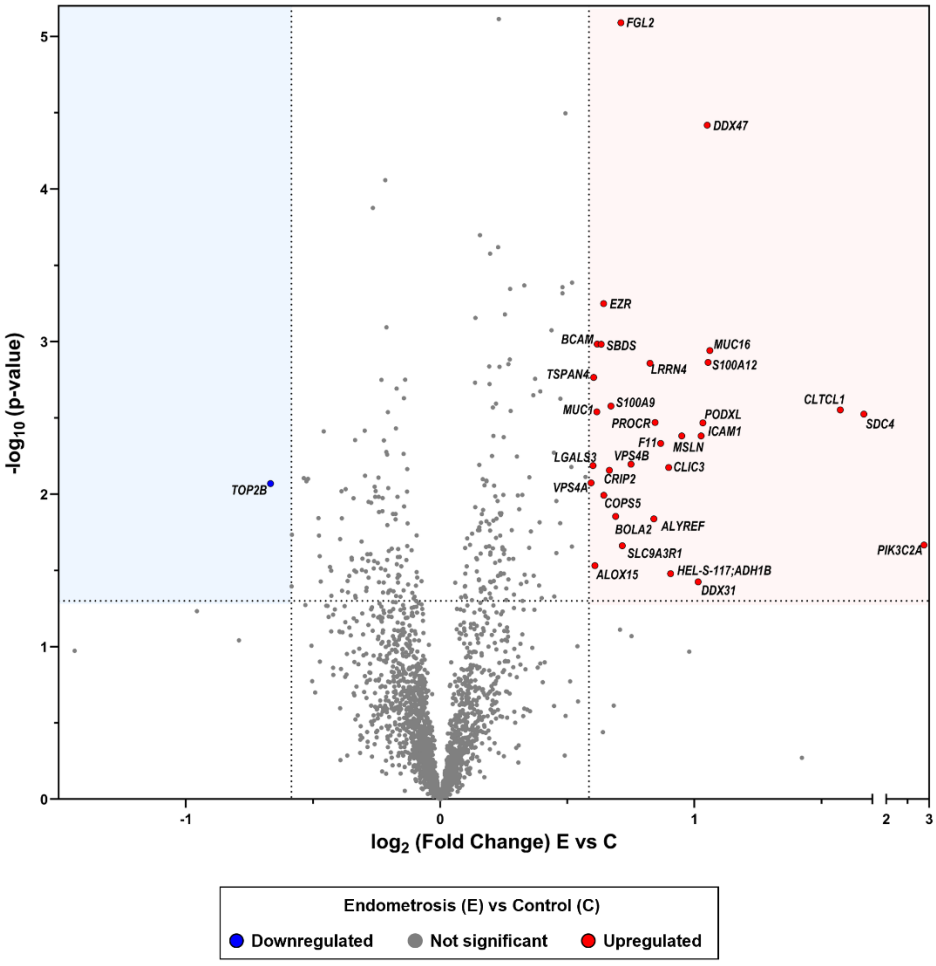

Figure 22. Volcano plot analysis of differentially abundant proteins in plasma EVs. Volcano plot analysis of DEPs in plasma EVs. Volcano plot illustrating the results of Student's t-test-based statistical analysis comparing plasma-derived EVs from patients with endometriosis (E) and controls (C). The x-axis represents log_2FC (E vs C), and the y-axis shows $-log10$(p-value) for each identified protein. Proteins meeting the predefined thresholds ($p < 0.05$ and $|log_2FC| \geq 0.585$) are highlighted in red (upregulated in E) and blue (downregulated in E), whereas non-significant proteins are shown in grey. Vertical dashed lines indicate the log_2FC cutoffs corresponding to a FC threshold of ±1.5, and the horizontal dashed line represents the statistical significance threshold ($p = 0.05$).

To further investigate the functional implications of the altered EV proteome, GO enrichment analysis was performed (Figure 23). Enrichment in BP revealed pathways associated with cell adhesion, immune regulation, and response to injury, aligning with known features of endometriosis pathophysiology. In the CC domain, enriched terms included EVs, plasma membrane, and vesicle-associated compartments, confirming the vesicular origin of the DEPs and their involvement in intercellular signalling pathways. Overall, the enriched GO categories indicate that plasma EV proteins are associated with immune-related and ECM-related biological processes.

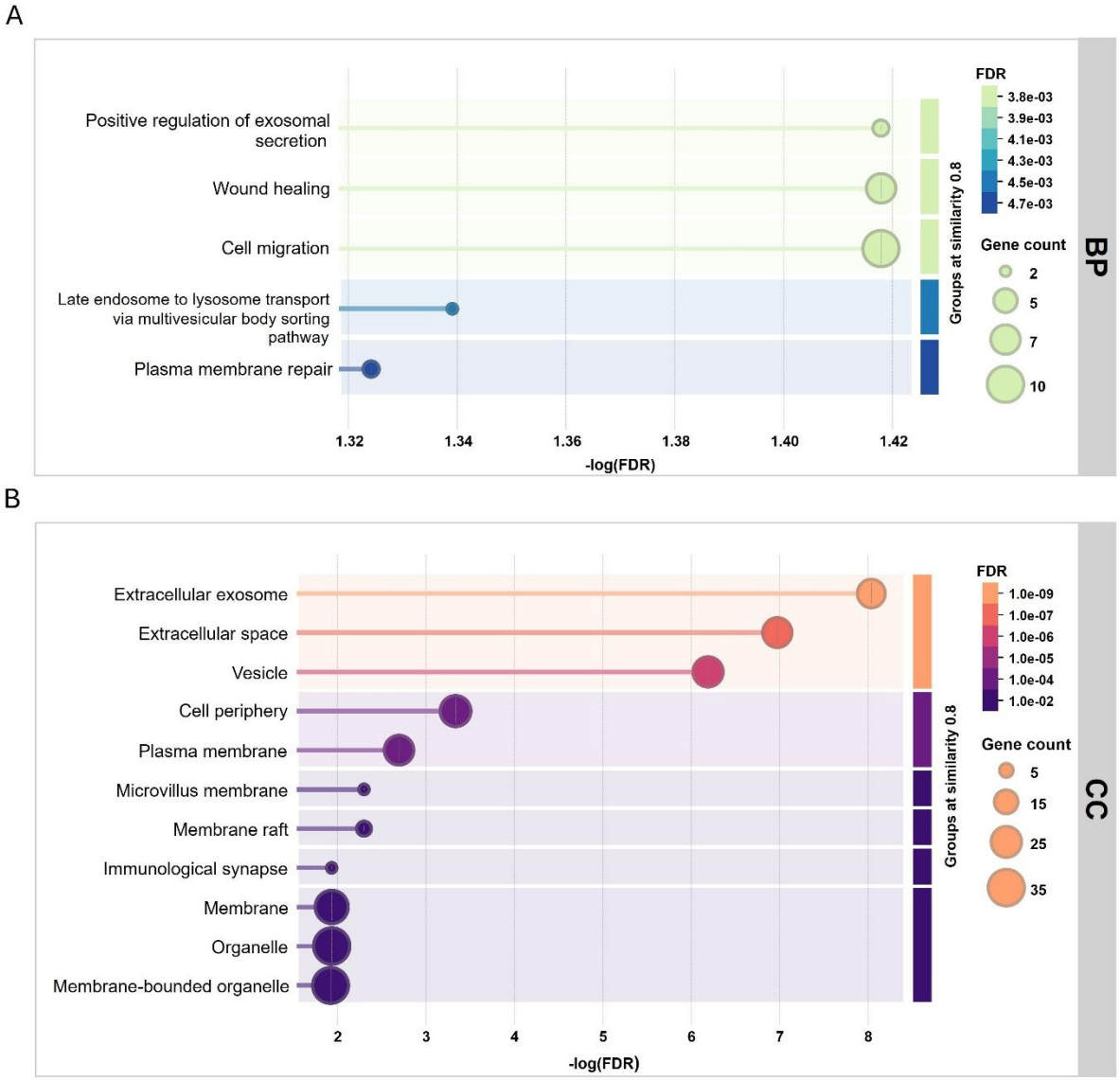

Figure 23. Functional enrichment analysis of DEPs identified in plasma-derived EVs from endometriosis patients. GO enrichment analysis was performed for DEPs identified in plasma EVs. (A) BP terms showing significant enrichment, including positive regulation of exosomal secretion, wound healing, cell migration, late endosome to lysosome transport via multivesicular body sorting pathway, and plasma membrane repair. (B) CC terms demonstrating strong enrichment in extracellular compartments, including extracellular exosome, extracellular space, vesicle, plasma membrane, membrane raft, and membrane-bound organelle. Bubble size represents gene count, and colour intensity corresponds to −log(FDR).

To investigate the potential functional interplay between DEPs in plasma-derived EVs from patients with endometriosis, a PPI network was constructed using the STRING database with a medium confidence threshold (score ≥ 0.400) (Figure 24). The network consisted of 36 nodes and 22 edges, corresponding to proteins with at least two high-confidence interactions. The observed number of interactions was significantly greater than expected by chance PPI enrichment (p-value < 0.001), indicating a biologically meaningful, non-random association pattern among these EV cargo proteins. Proteins were visualized according to their differential expression, with red halos indicating upregulation and blue halos indicating downregulation in endometriosis.

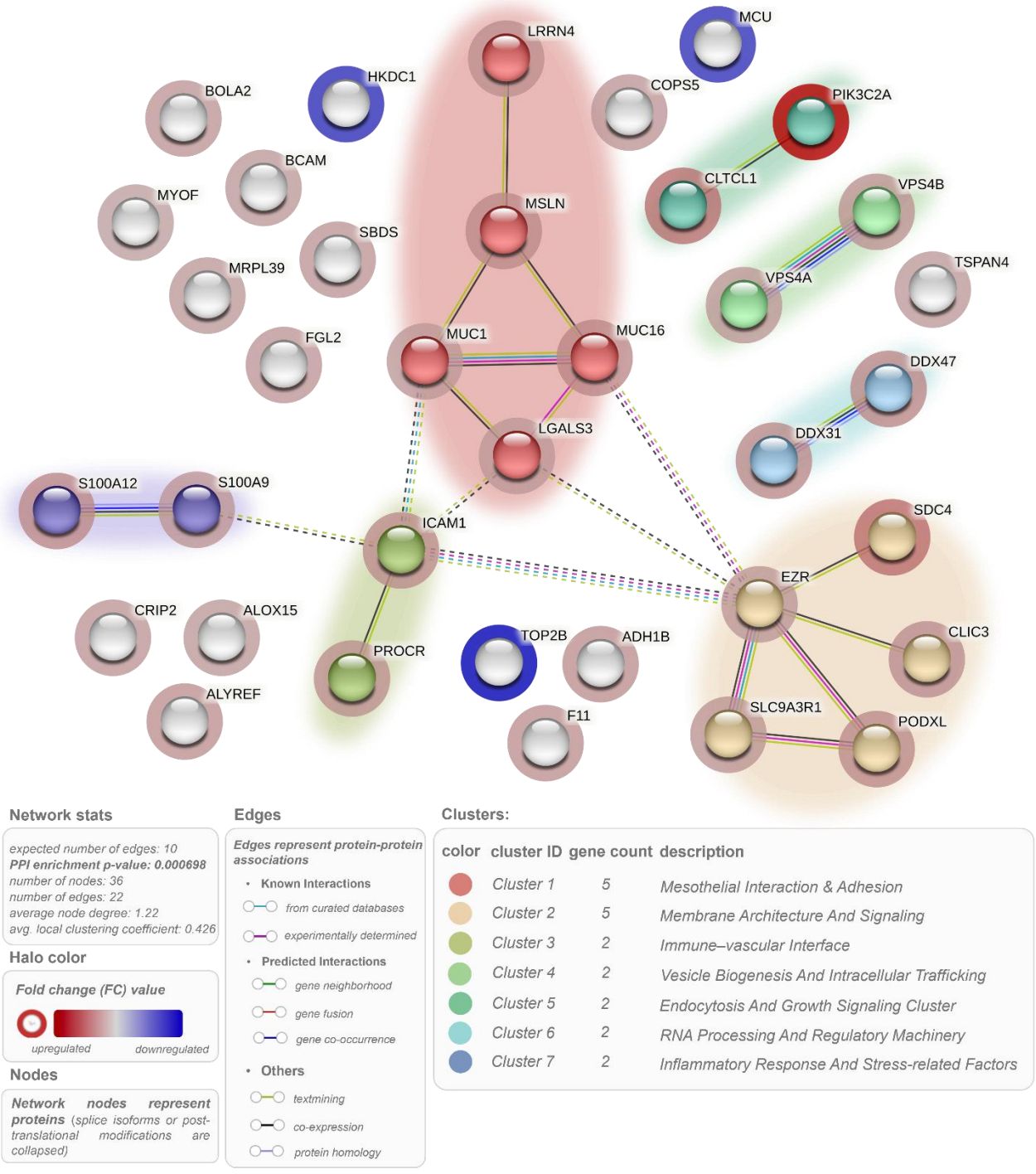

Figure 24. PPI network of DEPs in plasma-derived EVs from endometriosis patients. A PPI network was constructed using the STRING database, with a medium confidence threshold of 0.400 and integrated prediction methods (genomic context: neighbourhood, gene fusion, and co-occurrence; along with co-expression, text mining, curated databases, and experimental data). A total of 36 nodes (representing genes encoding functional proteins) were identified, with a PPI enrichment of p-value < 0.001, indicating a significantly non-random interaction pattern. Nodes are colour-coded based on fold change: upregulated proteins are highlighted in red and downregulated in blue. Only proteins with at least two interactions are displayed. PPI clusters were identified using the MCL with an inflation parameter of 2.8, allowing the delineation of moderately interconnected functional modules. Dashed lines represent inter-cluster connections. 7 functional clusters were defined and manually annotated based on dominant biological processes, including mesothelial adhesion, membrane remodelling, immune-vascular interactions, vesicle biogenesis, endocytosis, RNA regulation, and inflammatory signalling.

Application of the MCL with an inflation parameter of 2.8 enabled the identification of 7 functional clusters, representing moderately interconnected modules of biological relevance. Manual curation and annotation of these clusters, informed by STRING-derived functional associations, revealed distinct biological themes. Cluster 1 (red) encompassed proteins involved in mesothelial interaction and adhesion, including mucins (mucin 1 (MUC1) and MUC16) and galectin-3 (LGALS3). Cluster 2 (yellow) included proteins linked to membrane architecture and signalling, such as ezrin (EZR), SDC4, and PODXL.

Clusters 3–7 captured additional functional dimensions: the immune–vascular interface (ICAM1, protein C receptor (PROCR)), vesicle biogenesis and trafficking (vacuolar protein sorting-associated protein 4A (VPS4A) and vacuolar protein sorting-associated protein 4B (VPS4B), ATPases associated with the ESCRT pathway), endocytosis and growth signalling (CLTCL1 and PIK3C2A), RNA regulation (DEAD-box helicase 41 (DDX41) and DDX47, members of the DEAD-box RNA helicase family), and inflammatory or stress-related responses represented by calcium-binding S100A proteins (S100A9 and S100A12). Inter-cluster interactions were denoted with dashed lines and illustrate the connectivity between individual functional modules within the EV protein interaction network.

To further contextualize these findings, KEGG pathway enrichment analysis was performed using an FDR threshold of 0.2 and a minimum of 2 proteins per pathway (Figure 25).

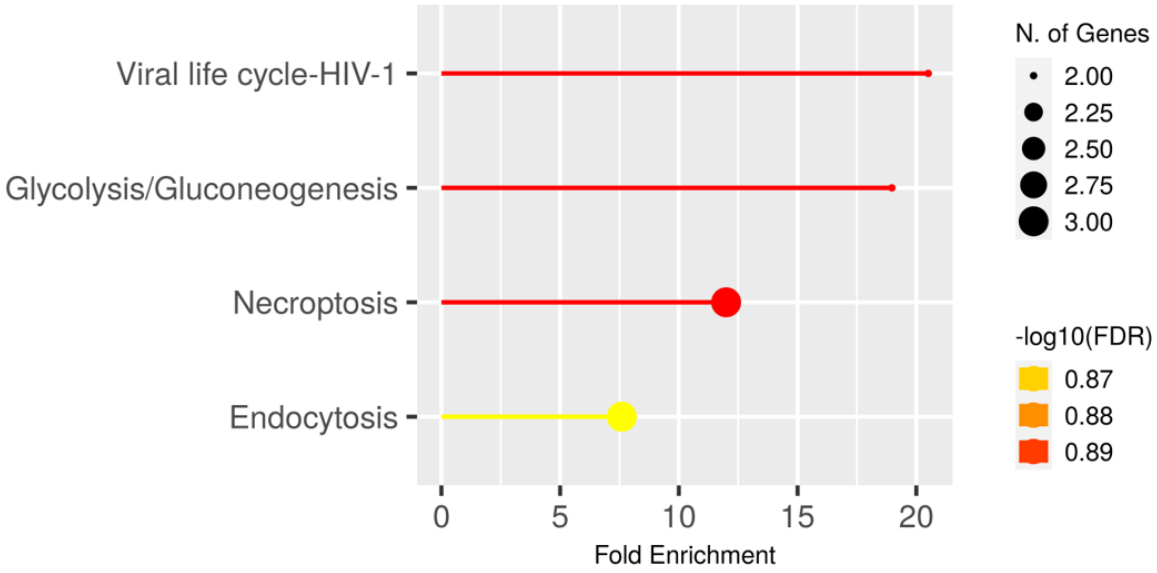

Figure 25. KEGG pathway enrichment analysis of differentially expressed proteins in plasma-derived EVs from endometriosis patients. Lollipop plot showing the top 4 enriched KEGG pathways ranked by fold enrichment. Dot size indicates the number of proteins mapped to each pathway, and dot colour corresponds to statistical significance ($-\log_{10}$(FDR)). Pathways were selected using an FDR cutoff of 0.2 and a minimum pathway size of 2 genes.

Four KEGG pathways were significantly enriched: necroptosis, endocytosis, glycolysis/gluconeogenesis, and viral life cycle-HIV-1. Necroptosis showed the highest fold enrichment (~15-fold), supporting its potential involvement in inflammatory cell death. Enrichment of the endocytosis pathway (~6.5-fold) corroborated vesicle-related findings from the PPI and GO analyses. The inclusion of metabolic and viral-related pathways may reflect stress-associated signalling or functional mimicry by EVs in chronic inflammatory states.

Collectively, these results indicate that plasma-derived EVs in endometriosis exhibit a distinct proteomic profile enriched in pathways associated with inflammation, membrane trafficking, and systemic signalling. These features underscore the potential of circulating EVs as accessible biomarkers and as mediators of disease-related communication beyond the peritoneal environment.

4.4. Comparative proteomic profiling and functional analysis of shared EV proteins from plasma and PF

To define robust molecular signatures of endometriosis, a comparative analysis of DEPs identified in EVs from both plasma and PF was performed, revealing 24 shared DEPs consistently dysregulated across both biofluids. The observed overlap suggests the presence of a core EV proteomic signature reflecting fundamental aspects of endometriosis-related immune modulation, tissue remodelling, and vesicle-mediated signalling across systemic and local compartments.

The graph in Figure 26 shows EV protein FC values in PF (black bars) and plasma (striped bars), highlighting statistically significant DEPs (coloured bars) and those with FDR < 0.05 (indicated by a yellow star). These proteins were manually classified into 6 functional categories based on their primary biological roles: oxidative stress, inflammatory/immune response, gene expression regulation, ECM remodelling, cell migration and invasion, and angiogenesis. This comparative bar plot of signed FC values across the 2 compartments illustrates both coordinated and compartment-specific expression patterns.

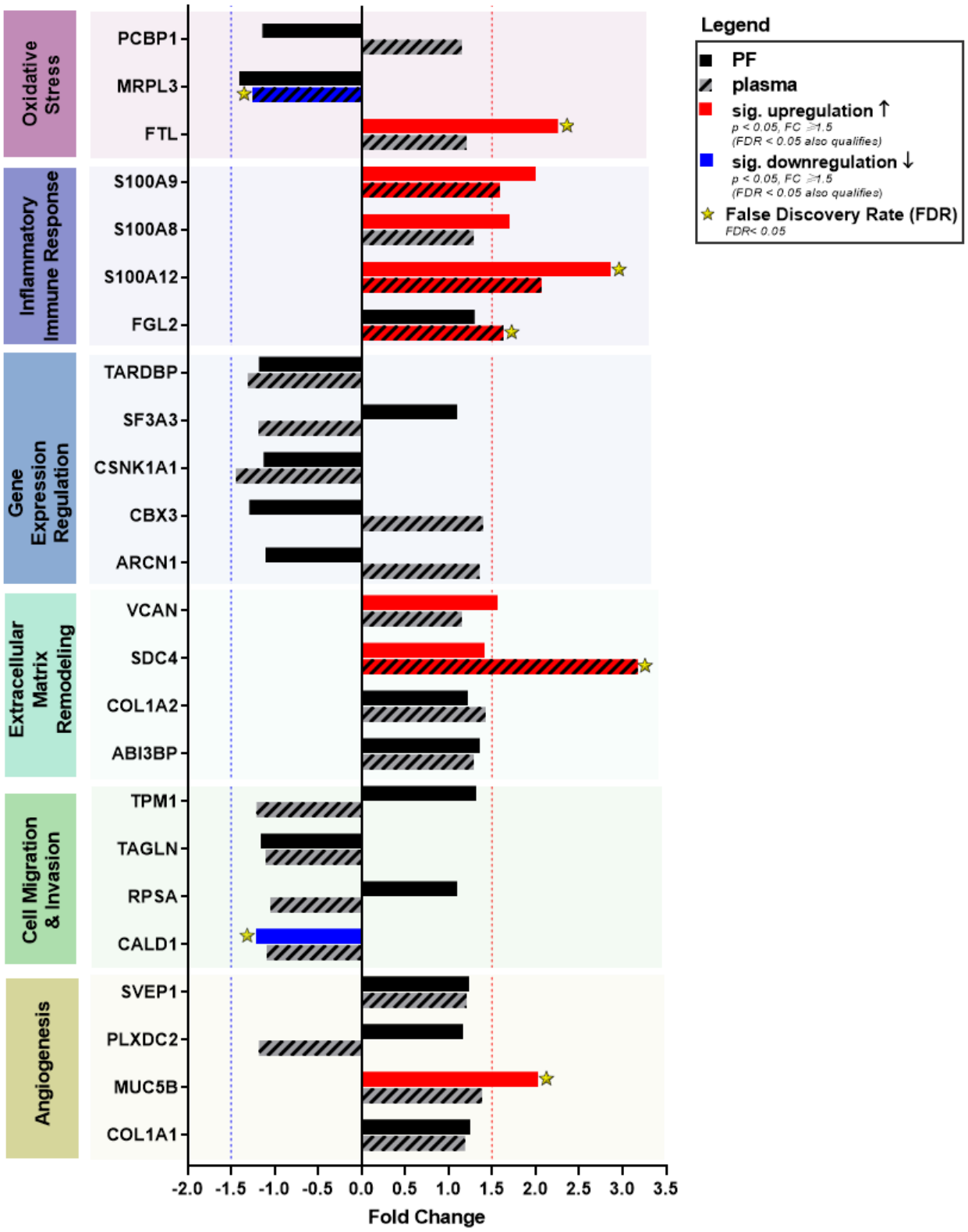

Figure 26. Shared DEPs in EVs from plasma and PF of endometriosis patients. Bar plot displaying signed FC values for 24 overlapping DEPs identified in EVs from both plasma (striped bars) and PF (solid bars). Proteins are grouped by functional category, including oxidative stress, immune response, gene expression regulation, ECM remodelling, cell migration, and angiogenesis. Statistically significant upregulation (red) or downregulation (blue) is indicated ($p < 0.05$), with asterisks denoting significance after multiple testing correction (FDR < 0.05). Vertical dashed lines indicate FC thresholds of ±1.5. Negative values indicate decreased abundance in endometriosis relative to controls.

Most shared proteins exhibited consistent expression profiles, however, 7 proteins showed opposite expression patterns between PF and plasma. For example, 3 out of 5 proteins related to gene expression regulation-namely splicing factor 3A subunit 3 (SF3A3), chromobox protein homolog 3 (CBX3) and archain 1 (ARCN1)-displayed opposite expression patterns in PF versus plasma, indicating localized regulation of gene expression machinery, possibly reflecting epigenetic or RNA-binding protein involvement in lesion development.

Notably, several inflammation-related proteins, including S100A8, S100A9, S100A12, and fibrinogen-like protein 2 (FGL2), were significantly upregulated in plasma-derived EVs, with similar trends observed in PF. These proteins are known mediators of immune cell recruitment and activation, suggesting a robust inflammatory signature in circulating EV cargo.Proteins involved in ECM remodelling (collagen type I alpha 2 chain (COL1A2), SDC4, and versican (VCAN)) were also consistently upregulated, particularly in plasma EVs. SDC4 showed the strongest upregulation (FC = 3.17 in plasma), underscoring its potential involvement in ECM reorganization and cell–matrix communication, both of which are critical in endometriotic lesion progression. By contrast, proteins associated with oxidative stress, such as mitochondrial ribosomal protein L3 (MRPL3) and caldesmon 1 (CALD1), exhibited significant downregulation in PF-derived EVs, while FTL showed strong upregulation in plasma. These findings suggest a complex redox environment, with both compensatory and stress-related protein signatures being exported via EVs.

Several proteins related to gene expression regulation, including CBX3, ARCN1, and TAR DNA-binding protein (TARDBP), displayed divergent expression between plasma and PF, potentially reflecting differential regulatory activity or EV cargo sorting in systemic versus local environments.Among all 24 shared DEPs, 5 reached statistical significance after FDR correction (FDR < 0.05) in at least one compartment, including SDC4, FGL2, S100A12, FTL, and CALD1.

These proteins span multiple functional domains and may represent robust and biologically relevant markers of EV-mediated signalling in endometriosis. Taken together, this manually curated functional classification highlights the diversity of EV cargo in endometriosis and points to a set of conserved, disease-relevant molecular signatures across both systemic and local biofluids.

To further characterize the functional roles of DEPs shared between PF- and plasma-derived EVs in endometriosis, a PPI network was constructed using the STRING database (Figure 27A). The resulting network consisted of 24 nodes and 23 edges, significantly more

than expected by chance (PPI enrichment p-value < 0.001 indicating biologically meaningful and non-random protein connectivity. Each node represents a protein identified in both PF and plasma EVs and differentially expressed between endometriosis patients and controls. FC values are shown as node halos, where red indicates upregulation and blue indicates downregulation in endometriosis. Inner pie charts within the nodes represent protein functional classification based on GO enrichment analysis. Edges represent predicted or known protein-protein interactions supported by curated databases, experimental data, gene co-expression, and other computational sources. Proteins clustered into functional modules involved in ECM organization, immune system regulation, and cell migration, aligning with the known hallmarks of endometriosis pathogenesis. Notably, ECM-related proteins such as SDC4, collagen type I alpha 1 chain (COL1A1), COL1A2, VCAN and CALD1 formed a tightly connected ECM signalling and tissue remodelling cluster, while inflammatory mediators like S100A8/A9 and FGL2 anchored a distinct immune-related module.

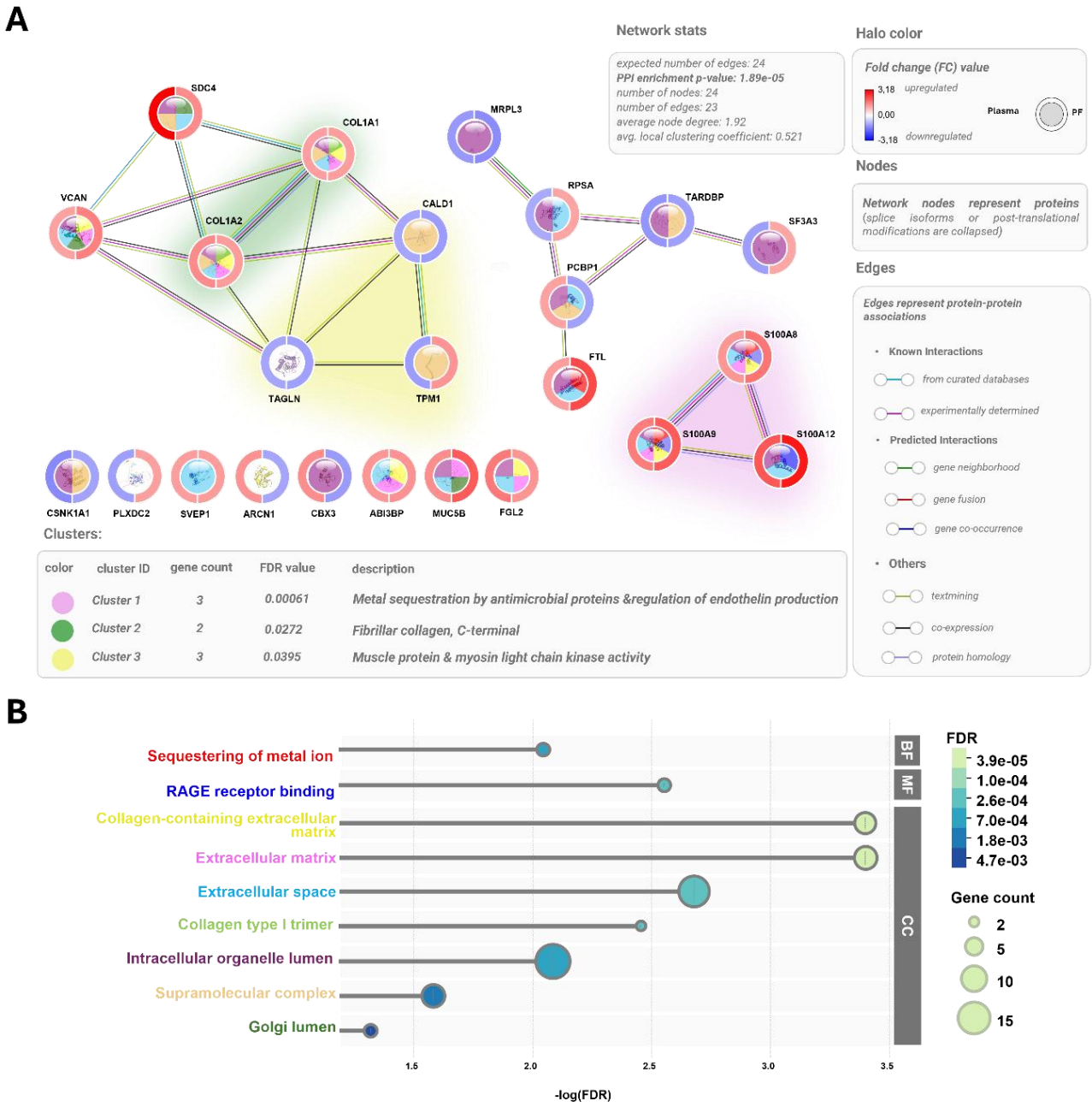

Figure 27. PPI network and GO enrichment of EV-derived proteins shared between PF and plasma in endometriosis. (A) STRING-based PPI network of DEPs identified in EVs from both PF and plasma. Nodes represent individual proteins; node halos indicate FC between endometriosis and control groups (red = upregulated, blue = downregulated). Inner pie charts reflect relative protein distribution between PF and plasma EVs. Edges represent known or predicted protein-protein associations based on curated databases, experiments, co-expression, gene neighbourhood, and other sources. (B) GO enrichment analysis for shared EV proteins, grouped by BP, MF, and CC terms. Dot size reflects the number of proteins annotated to each term, and colour intensity indicates statistical significance (adjusted p-value). The colours of GO term labels correspond to the central node colours of the respective clusters identified in the PPI network, linking enriched functional categories to their associated interaction modules. Notably enriched terms include ECM organization, ECM structural constituent, collagen-containing ECM, and Golgi lumen.

To gain further insight into the biological relevance of shared EV-derived DEPs, GO enrichment analysis was performed across 3 domains: BP, MF, and CC (Figure 27B).

Functional enrichment analysis further confirmed localization of many proteins to the extracellular space and collagen-rich matrices, underscoring their relevance to lesion establishment and immune modulation. In the BP category, the most significantly enriched term was ECM organization, suggesting that EV proteins commonly participate in structural tissue remodelling processes implicated in endometriosis. Additional enriched terms included cell-matrix adhesion and collagen fibril organization, both of which are relevant to the fibrotic and adhesive features of the disease. In the MF category, RAGE receptor binding was identified, probably on basis of the S100 proteins, known RAGE ligands, responsible for the activation of fibrosis-related and inflammation-associated pathways. In the CC category, many DEPs were primarily localized to or function within the ECM, consistent with their enrichment in EVs and relevance to lesion formation, fibrosis, and immune cell trafficking. Enrichment in collagen-containing ECM and Golgi lumen, reflects the involvement of EV-associated DEPs in ECM formation and secretion. The enrichment in Golgi lumen components may indicate altered protein processing or trafficking in endometriosis-derived EVs.

Reactome pathway enrichment analysis was performed to further explore and depict in a different way the functional roles of DEPs derived from EVs that are shared between PF and plasma in endometriosis. The analysis revealed again significant enrichment of immune-related and ECM-associated pathways, further confirming that EV-associated proteins commonly found in both fluids may contribute to inflammatory and tissue-remodelling processes characteristic of endometriosis (Figure 28).

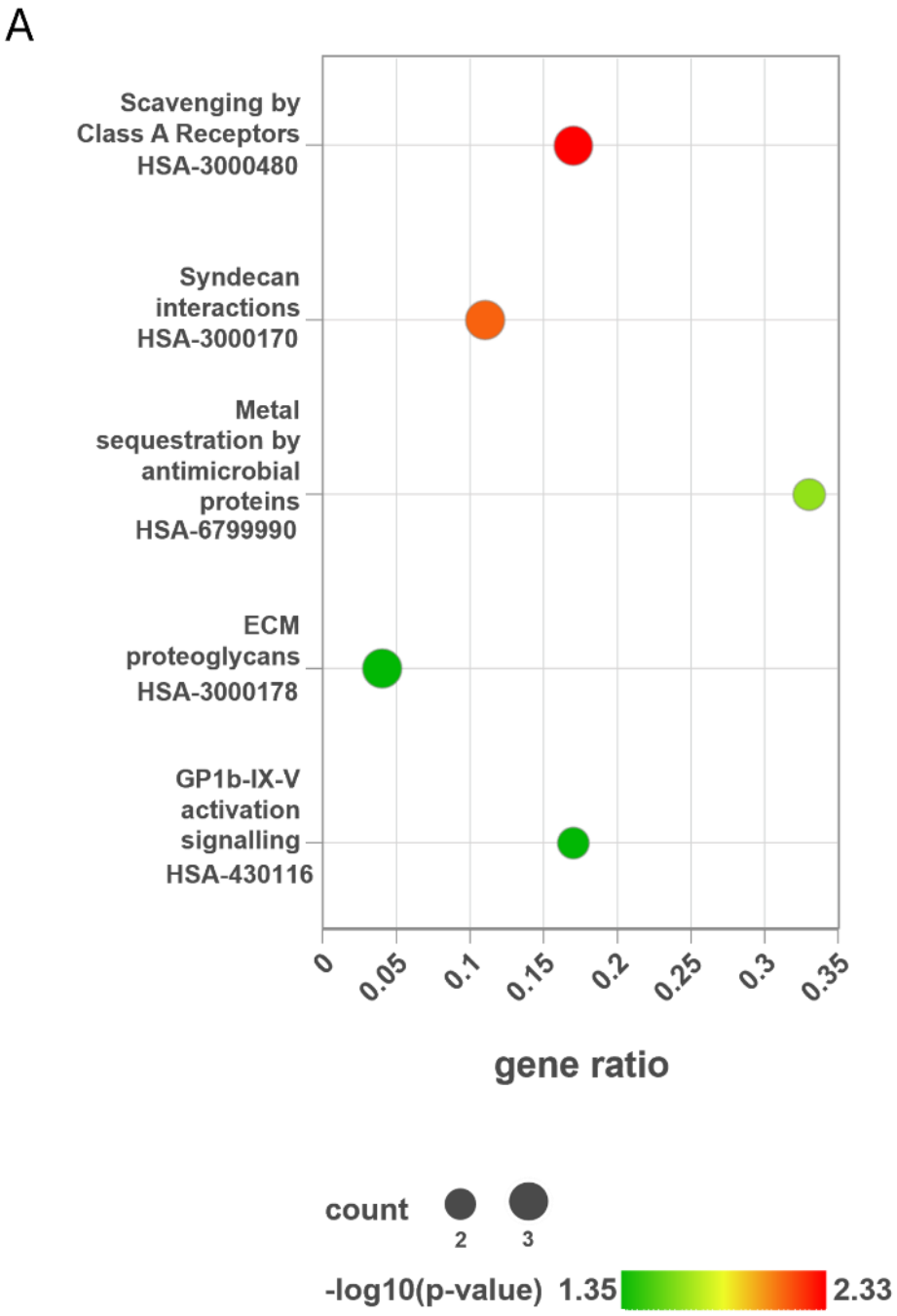

Figure 28. Reactome pathway enrichment analysis and functional mapping of EV-derived proteins shared between PF and plasma from endometriosis patients. (A) Bubble plot showing significantly enriched Reactome pathways among DEPs present in EVs isolated from both PF and plasma. The x-axis indicates the gene ratio (proportion of DEPs within each pathway), while the y-axis lists the enriched pathways. Bubble size reflects the number of identified proteins in each pathway, and colour represents statistical significance ($-\log_{10}$(p-value)), with red indicating the most significant enrichment.

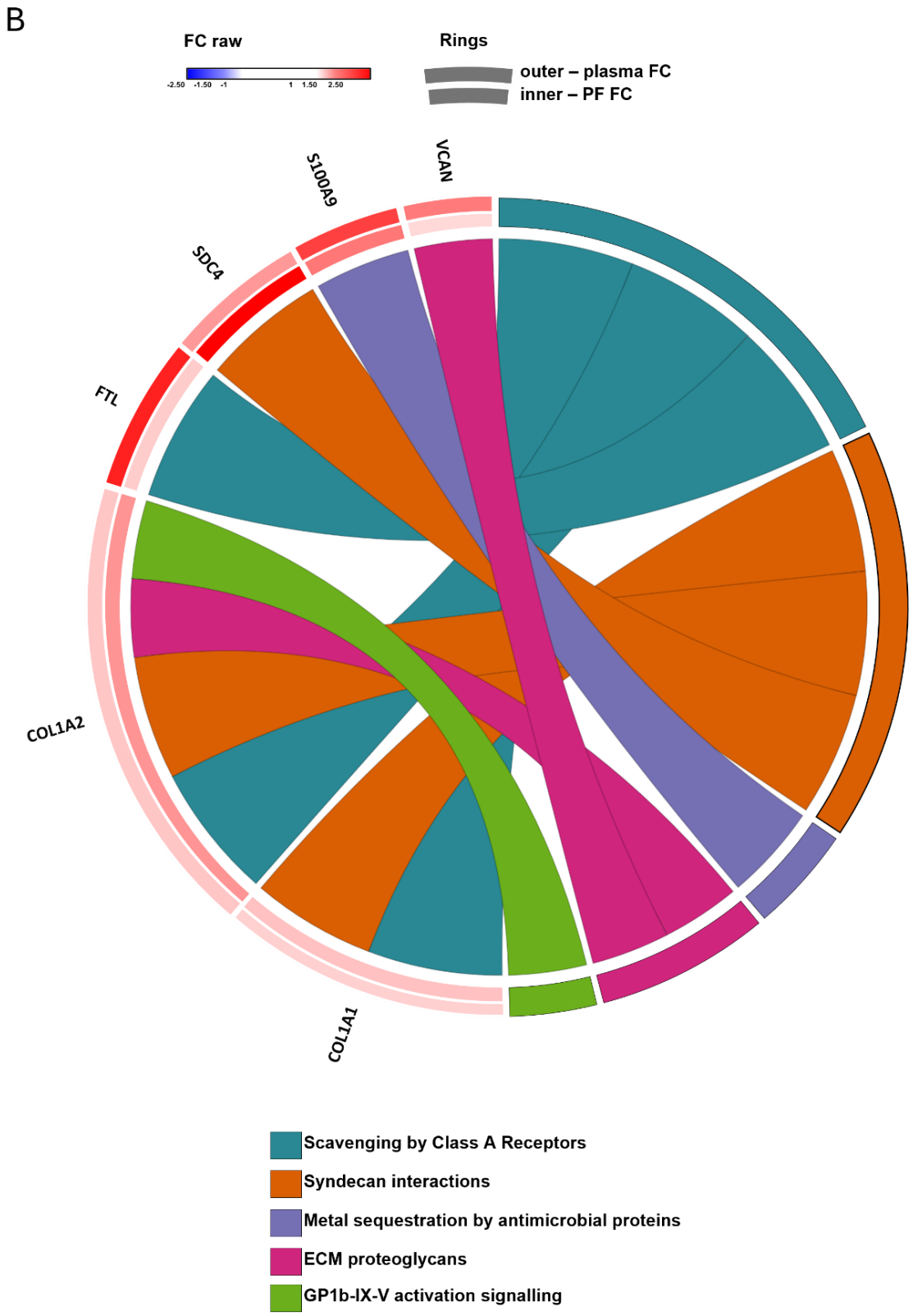

Figure 28. Continued. (B) Chord diagram illustrating functional relationships between differentially expressed EV-derived proteins shared between PF and plasma, and enriched Reactome pathways. Pathways are colour-coded and include: Metal Sequestration by Antimicrobial Proteins, Scavenging by Class A Receptors, Syndecan Interactions, ECM Proteoglycans, and GP1b-IX-V Activation Signalling. FC represents differential expression between endometriosis patients and controls: red = upregulated in endometriosis, blue = downregulated. The inner ring shows FC-based expression of DEPs in PF EVs, and the outer ring shows expression in plasma EVs.

The bubble plot (Figure 28A) displays the most significantly enriched Reactome pathways, ranked by gene ratio (i.e., the proportion of shared DEPs mapped to each pathway). Bubble size indicates the number of DEPs per pathway, and colour intensity corresponds to enrichment significance (−log10(p-value)), with red representing the most significant enrichment. Specifically, Scavenging by Class A Receptors (HSA-3000480) and Syndecan Interactions (HSA-3000170) emerged as top-ranked pathways, driven by the presence of innate immune proteins (S100A8/A9/A12) and matrix-associated molecules (SDC4, VCAN, COL1A2). Other significantly enriched pathways included Metal Sequestration by Antimicrobial Proteins (HSA-6799990), also involving S100 proteins, which are critical for neutrophil-mediated antimicrobial defence and indicate a role in host defence mechanisms.

The moderate significance of enrichment of DEPs in the ECM Proteoglycans pathway (HSA-3000178) suggests potential dysregulation of extracellular matrix components—collagens, glycoproteins, and integrins—commonly implicated in lesion adhesion and fibrotic transformation. In contrast, a lower, borderline enrichment ($p \approx 0.05$) observed for the glycoprotein Ib–IX–V (GPIb–IX–V) activation signalling pathway (HSA-430116) points to a possible involvement of EVs in platelet activation and vascular–inflammatory processes in endometriosis. In summary, enrichment in innate immunity, ECM organisation, and cell surface receptor interactions supports a multifaceted role for EV-associated proteins in inflammation, cell migration, and tissue remodelling in endometriosis.

To further characterize functional associations between DEPs and pathways, a chord diagram was generated (Figure 28B). Only proteins identified in EVs from both PF and plasma were included. The diagram highlights strong functional clustering among DEPs associated with Metal Sequestration by Antimicrobial Proteins, Scavenging by Class A Receptors, Syndecan Interactions, ECM Proteoglycans, and glycoprotein Ib–IX–V (GPIb–IX–V) activation signalling pathways.

The inner ring represents expression in PF EVs, while the outer ring corresponds to plasma EVs. FC values indicate differential expression between endometriosis patients and controls, with red indicating upregulation in endometriosis and blue indicating downregulation. The chord plot reveals the overlap of individual EV proteins across multiple pathways. Several proteins act as hubs, connecting multiple enriched pathways, indicating their pleiotropic roles in the endometriosis microenvironment. The collagen type I proteins, COL1A1 and COL1A2, are for example strongly linked to both scavenger receptor, syndecan interactions and ECM proteoglycans pathways -reinforcing their importance in cell-matrix interactions and signal transduction. These findings highlight the complex functional

landscape of EV cargo and underscore their potential role in orchestrating immune modulation, tissue remodelling, and lesion progression in endometriosis.

4.5. IFCM analysis of EV-associated proteins

In addition to global MS-based proteomic profiling of EVs, targeted multiparametric analysis of selected EV-associated surface proteins was performed using imaging flow cytometry (IFCM). This complementary approach enabled characterization of the molecular phenotype of EVs at the single-vesicle level and quantitative assessment of selected markers in both systemic (plasma) and local (PF) compartments.

Multiparametric EV phenotyping was performed using an ImageStreamX Mark II imaging flow cytometer (Amnis/Luminex). IFCM represents a high-resolution method recommended for EV analysis in limited sample volumes and enables simultaneous assessment of particle size, scatter properties, and expression of multiple biomarkers on individual vesicles directly in the original biofluid. This platform allows detection and quantification of particles within defined size, scatter, and fluorescence ranges. EV quantification was based on particle count (number of recorded events per gated population per mL of sample), mean fluorescence intensity (MFI), and the percentage of signal-positive events for each analysed marker.

All IFCM analyses were performed in cooperation with Prof. Bernd Giebel (University of Duisburg–Essen, Germany), ensuring standardized acquisition parameters and gating strategies

4.5.1 Perioperative plasma cohort: assay establishment and biological validation

To establish and validate the IFCM assay, plasma samples from an independent patients cohort, collected during another co-authored study[121], were used. The samples were collected from patients with endometriosis before and 48 h after laparoscopic surgical treatment (n = 15 per time point) (Figure 29).

Quantitative comparison revealed a significant reduction in multiple EV-associated surface markers following surgical removal of endometriotic lesions. As shown in Figure 29A, the canonical EV markers CD81 and CD63 were significantly decreased 48 h after surgery, whereas CD9 did not exhibit a statistically significant change. A strong reduction was observed among immune-associated markers (Figure 29B), including CD14, CD16, CD206, HLA-ABC, and HLA-DR, with the most pronounced decrease detected for HLA-DR. Markers associated with platelet- and erythrocyte-derived EVs (CD41, CD61, and CD235a)

were also significantly reduced 48 h post-operatively (Figure 29C). In addition, the adhesion-related markers CD44 and CD82 (Figure 29D) demonstrated significant decreases, with CD44 showing one of the strongest effects across all analysed proteins. The epithelial-associated marker CD227 (MUC1) was likewise significantly reduced in plasma samples collected 48 h after surgery (Figure 29E).

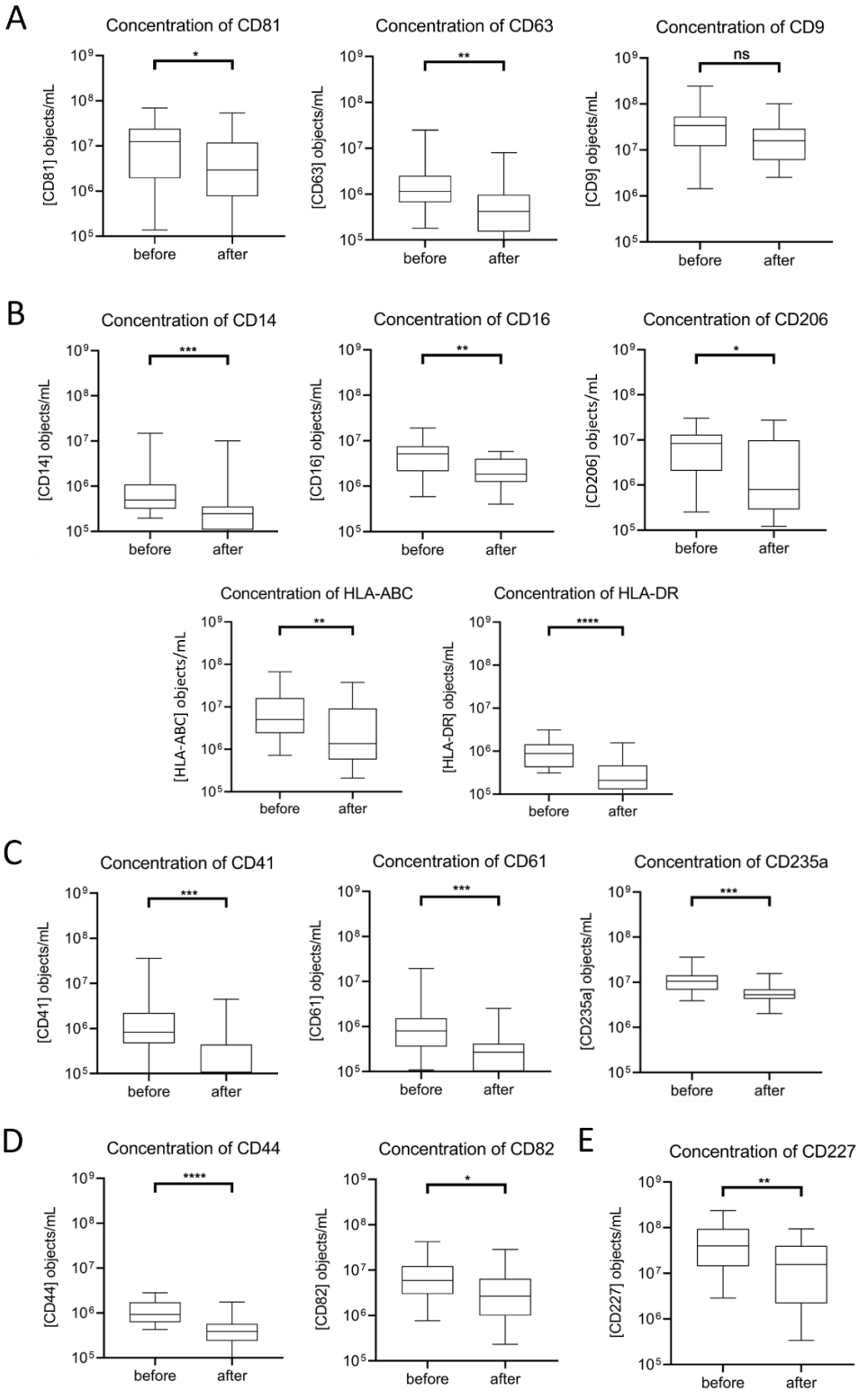

Figure 29. IFCM analysis of plasma EVs before and after laparoscopic surgery in endometriosis patients. Multiparametric IFCM analysis of EV-associated surface markers in plasma samples collected from endometriosis patients before (n = 15) and after (n = 15) laparoscopic surgical treatment. EV concentration (objects/mL) of marker-positive vesicles is presented as boxplots showing the median and Tukey whiskers (1.5× IQR). Statistical outliers were identified and excluded prior to analysis. (A) Canonical EV markers: CD81, CD63, CD9. (B) Immune-associated markers: CD14, CD16, CD206, HLA-ABC, HLA-DR. (C) Platelet- and erythrocyte-associated markers: CD41, CD61, CD235a. (D) Adhesion-related markers: CD44, CD82. (E) Epithelial-associated marker: CD227 (MUC1). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as indicated.

Importantly, no significant differences in total EV particle concentration were observed between samples collected before and 48 h after surgery, as assessed by NTA using both scatter and fluorescence modes (Figure 30A–B), indicating that the observed changes were not associated with a global reduction in EV abundance.

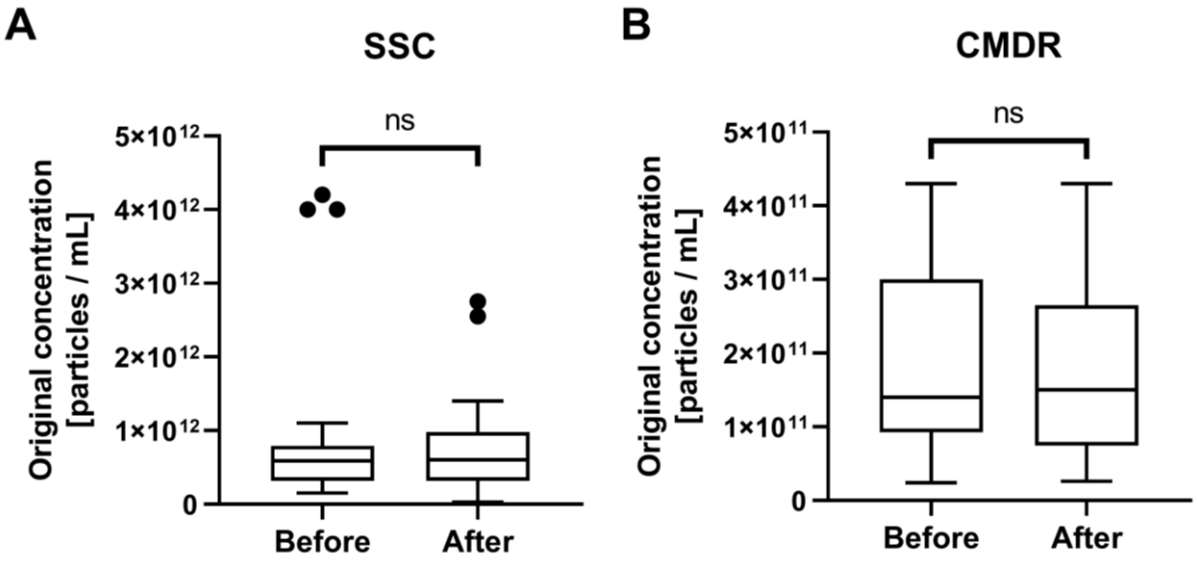

Figure 30. Quantitative analysis of plasma-derived EV concentration before and 48 hours after laparoscopic surgery in endometriosis patients. (A) Original particle concentration (particles/mL) measured in scatter mode (side scatter, SSC). (B) Particle concentration measured in fluorescence mode using CellMask™ Deep Red (CMDR). Data are presented as boxplots showing the median and Tukey whiskers (1.5 × IQR). Statistical comparisons between groups were performed using the non-parametric Wilcoxon matched-pairs test. n = 15. ns, not significant.

Overall, the analysed plasma cohort demonstrated coordinated early modulation of multiple functional classes of EV-associated proteins within 48 h following surgical intervention, confirming the technical robustness and sensitivity of IFCM-based EV phenotyping as well as the potential clinical significance of EV-related plasma biomarkers.

4.5.2 IFCM analysis of PF and plasma EVs

Following assay optimization and validation, the same IFCM antibody panel was applied to a small independent cohort comprising plasma and PF samples from 30 patients with endometriosis and 16 control subjects. Of note, these samples were not subjected to the previously described MS-based profiling.

Importantly, in contrast to the previously described IFCM experiment, this time the EVs were not isolated prior to analysis. Instead, multiparametric IFCM measurements were performed directly in original plasma and PF samples, enabling the detection and quantification of marker-positive particles in their native biological context.

In PF samples, multiple markers demonstrated statistically significant differences between endometriosis patients and controls (Figure 31). As shown in Figure 31A, antigen presentation-associated markers HLA-ABC and HLA-DR were significantly elevated in endometriosis. Notably, HLA-DR had previously been identified as a DEP in the proteomic analysis of PF-derived EVs, providing cross-platform validation of this finding. In contrast, HLA-ABC was detected in the global proteomic dataset but was not classified as a DEP.

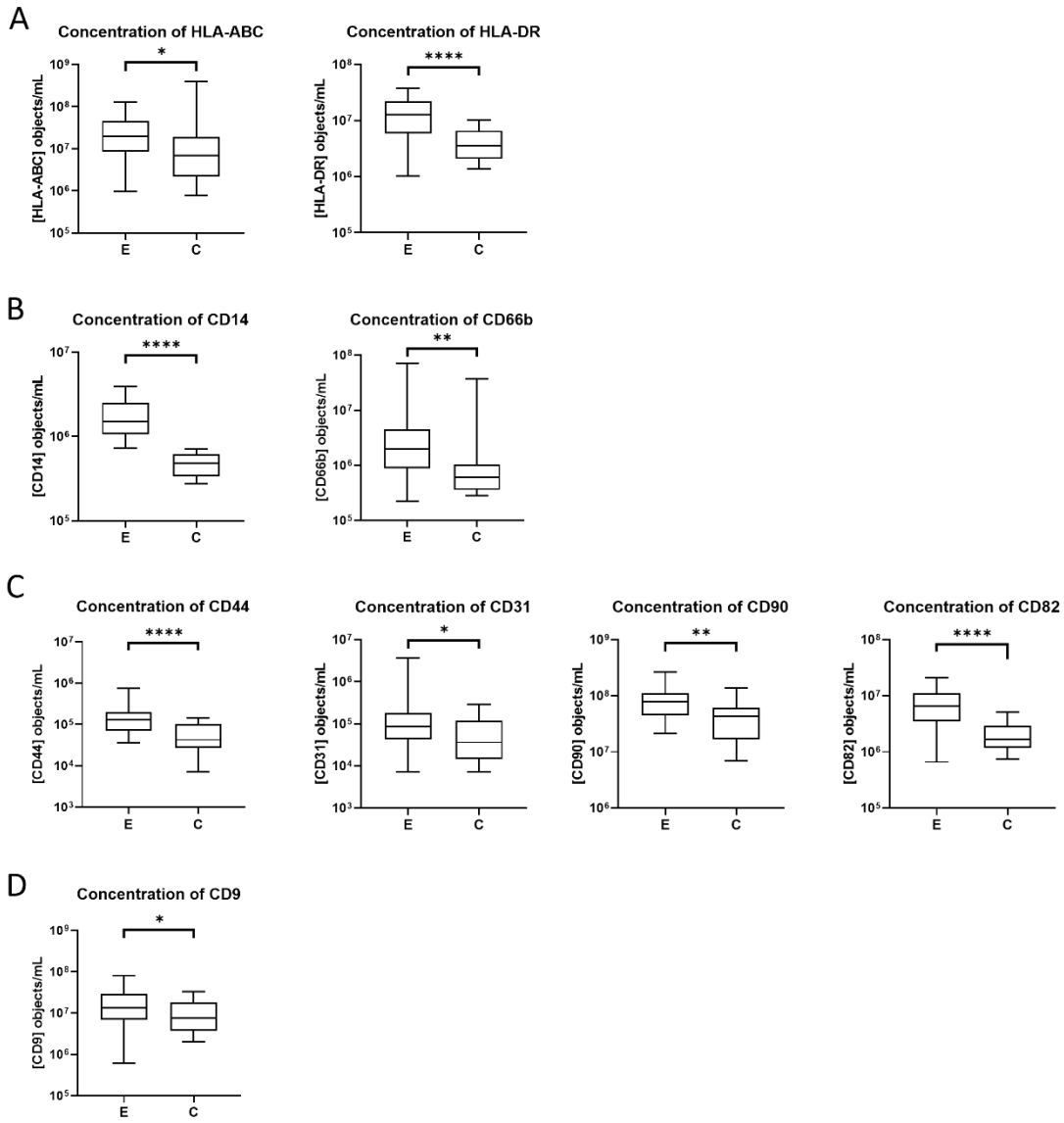

Figure 31. IFCM analysis of marker-positive particles directly in PF samples from endometriosis patients and controls. Multiparametric IFCM analysis of selected surface markers was performed directly in original PF samples obtained from endometriosis patients (E, n = 30) and control subjects (C, n = 16), without prior EV isolation. The concentration (objects/mL) of marker-positive particles is presented as boxplots showing the median and Tukey whiskers (1.5× IQR). Statistical outliers were identified and excluded prior to analysis.(A) Antigen presentation markers: HLA-ABC, HLA-DR. (B) Innate immune-associated markers: CD14, CD66b. (C) Stromal and vascular-associated markers: CD31, CD90, CD44, CD82. (D) Canonical EV marker: CD9. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as indicated.

Figure 31B illustrates significant differences in innate immune-associated markers CD14 and CD66b, both increased in endometriosis samples. CD14 had been detected in the global proteomic dataset but did not reach differential abundance criteria, whereas CD66b was not identified as a DEP in the proteomic analysis. Markers related to stromal and vascular compartments, including CD31, CD90, CD44, and CD82 (Figure 31C), also showed significant modulation in PF samples from endometriosis patients. Among these, CD44 had been detected in the proteomic dataset but was not classified as differentially abundant, while CD31, CD90, and CD82 were not identified as DEPs. Finally, the canonical EV marker CD9 (Figure 31D) demonstrated a significant difference between groups. CD9 was detected in the global proteomic dataset but did not meet criteria for differential abundance.

In contrast to PF, a more restricted set of markers differentiated endometriosis patients from controls in plasma samples from the same cohort (Figure 32). As shown in Figure 32A, the canonical EV marker CD81 demonstrated a significant difference between groups. CD81 had previously been identified as a DEP in the proteomic analysis of plasma-derived EVs, indicating concordance between IFCM-based phenotyping and MS-based profiling. The epithelial-associated marker CD227 (MUC1) (Figure 32B) also showed a significant difference between endometriosis and control samples and had been classified as a DEP in plasma-derived EVs. In contrast, the immune checkpoint-associated marker PD-L1 (Figure 32C) demonstrated statistically significant differences in IFCM analysis but had not been identified as a differentially abundant protein in the proteomic dataset.

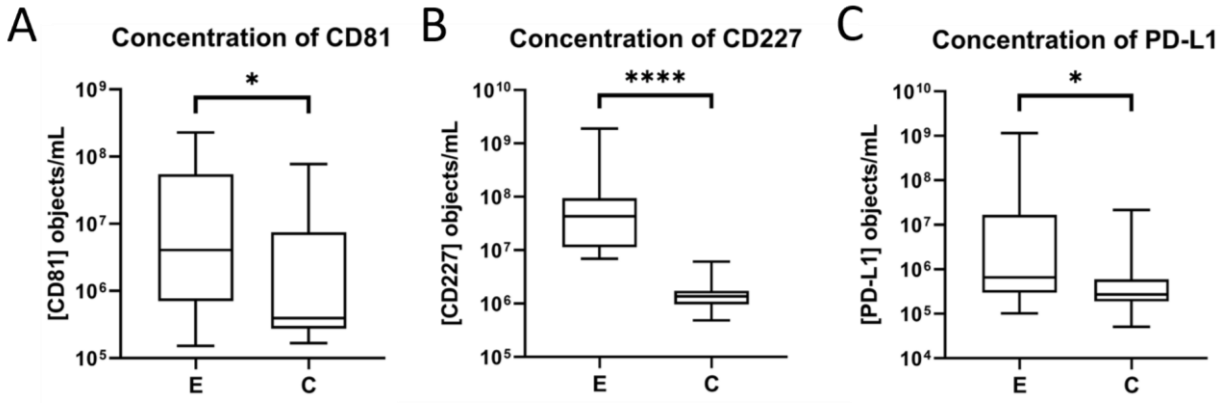

Figure 32. IFCM analysis of marker-positive particles in plasma samples from endometriosis patients and controls. Multiparametric IFCM analysis of selected surface markers was performed directly in original plasma samples obtained from endometriosis patients (E, n = 30) and control subjects (C, n = 16), without prior EV isolation. The concentration (objects/mL) of marker-positive particles is presented as boxplots showing the median and Tukey whiskers (1.5× IQR). Statistical outliers were identified and excluded prior to analysis. (A) Immune checkpoint-associated marker: PD-L1. (B) Epithelial-associated marker: CD227 (MUC1). (C) Canonical EV marker: CD81. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as indicated.

Overall, IFCM analysis in plasma revealed partial overlap with proteomic findings, with concordant modulation of CD81 and CD227, while also identifying PD-L1 as an

additional marker detectable through targeted single-particle phenotyping. Table 6 summarizes the cross-platform comparison of EV-associated proteins identified by IFCM and MS.

Table 6. Integrated cross-platform comparison of EV-associated proteins identified by IFCM and MS.

Functional group	Protein	IFCM detected (sig.)	MS detected	MS DEP
Antigen presentation	HLA-ABC	PF	PF + Plasma	↑ Plasma (HLA-A)
	HLA-DR	PF	PF + Plasma	↑ PF (HLA-DRB1)
Tetraspanins / EV markers	CD9	PF	PF + Plasma	-
	CD81	Plasma	PF + Plasma	↑ Plasma
Innate immunity / myeloid	CD14	PF	PF + Plasma	↑ Plasma
Adhesion / stromal	CD44	PF	PF + Plasma	-
	CD82	PF	PF + Plasma	-
	CD31	PF	PF	-
	CD90 (THY1)	PF	PF + Plasma	-
Epithelial-associated	CD227 (MUC1)	Plasma	PF + Plasma	↑ Plasma

5. DISCUSSION

5.1. Rationale and methodological framework for EV-based biomarker discovery in endometriosis

Endometriosis remains a diagnostically challenging disease, frequently associated with prolonged diagnostic delay and the need for invasive surgical confirmation. In this context, the identification of reliable, minimally invasive biomarkers represents a major unmet clinical need. EVs, as membrane-enclosed nanoparticles carrying proteins, lipids, and nucleic acids that reflect the physiological and pathological state of their cells of origin, have emerged as promising candidates in biomarker discovery. Owing to their relative stability in biological fluids and their capacity to protect molecular cargo from enzymatic degradation, EVs are an attractive source of clinically relevant information. In endometriosis, both plasma and PF constitute biologically relevant sources of EVs. Plasma reflects systemic alterations, whereas PF directly represents the local inflammatory and immunomodulatory microenvironment of the peritoneal cavity.

Accordingly, the present study was designed to comprehensively characterise and compare EVs isolated from plasma and PF of patients with endometriosis, with the overarching aim of performing quantitative proteomic analysis and identifying disease-associated differences in EV protein composition. A key objective was to determine whether EV-derived proteomic signatures reflect both local peritoneal pathology and systemic manifestations of the disease. At the same time, recognising that reliable proteomic profiling fundamentally depends on the quality of the starting material, particular emphasis was placed on rigorous EV isolation and comprehensive characterisation. The goal was to ensure high-purity and well-defined vesicle preparations to minimise technical variability and contamination that could obscure subtle yet biologically relevant differences in protein abundance. This would guarantee that, subsequent proteomic findings can be confidently attributed to genuine alterations in EV cargo rather than methodological artefacts.

Both plasma and PF are highly complex biological matrices. Plasma contains abundant albumin, immunoglobulins, and lipoproteins (notably apolipoprotein A1 (ApoA1)- and apolipoprotein B (ApoB)-associated particles), which represent major confounders in EV proteomics due to their high dynamic range and propensity for co-isolation[130, 131]. Although PF contains lower absolute concentrations of circulating proteins compared with plasma, it is enriched in inflammatory mediators, soluble immune factors, proteases, and cell-derived debris, all of which may interfere with downstream molecular analyses [65, 132]. Therefore,

careful optimisation of EV isolation was essential to ensure reliable discrimination between vesicle-associated cargo and non-vesicular contaminants [76].

SEC was selected as the primary isolation strategy based on its favourable balance between recovery and specificity, as well as its compatibility with downstream MS workflows. In complex biological matrices such as plasma, complete separation of EVs from co-isolated particles-including lipoproteins and abundant soluble proteins-remains inherently challenging due to the extreme dynamic range of plasma proteins and the physicochemical overlap between EVs and non-vesicular particles [130, 133].

Comparative analyses of commonly used isolation techniques have demonstrated that SEC generally provides higher particle-to-protein ratios than ultracentrifugation, indicating improved purity and reduced contamination by albumin and other high-abundance plasma proteins[134, 135]. Importantly, SEC separates particles according to hydrodynamic radius under mild, non-denaturing conditions, thereby preserving vesicle integrity and minimising structural disruption. SEC-driven removal of most soluble plasma proteins is essential in proteomics-driven studies, where abundant plasma proteins may otherwise suppress detection of low-abundance EV cargo in MS-based workflows[130]. Recent methodological evaluations further support SEC-based workflows as a reproducible and proteomics-compatible approach, although complete elimination of lipoprotein contamination, particularly ApoB-containing particles, remains technically challenging [76, 133, 136]. Thus, SEC represents a pragmatic compromise between purity and recovery in plasma EV studies.

Our distinct elution profiles observed in the present study for both plasma- and PF-derived EVs were characterised by a clear particle concentration peak in fraction 5 and a delayed increase in total protein levels in later fractions. They are consistent with previously reported SEC fractionation patterns for plasma-derived EVs, in which vesicle-enriched fractions elute earlier than the majority of soluble plasma proteins, resulting in higher particle-to-protein ratios in early fractions [134, 135]. Comparable elution behaviour in both plasma and PF further demonstrates that SEC provides robust fractionation across biologically distinct matrices. While plasma exhibited overall higher particle and protein concentrations, consistent with its systemic origin and broader proteomic complexity, separation efficiency was preserved in both fluids, supporting the suitability of SEC for proteomics-oriented EV biomarker studies in endometriosis.

To ensure methodological standardisation and reproducibility, commercially available qEV SEC columns (Izon Science) were employed for EV isolation. The use of pre-packed, quality-controlled columns minimises operator-dependent variability, which is particularly

important in comparative clinical and quantitative proteomics studies. The 70 nm configuration was selected specifically from the available pore-size series (20 nm, 35 nm, and 70 nm) to prioritise isolate purity over maximal particle recovery. The 70 nm series preferentially enriches vesicle-sized particles while delaying smaller soluble proteins and lipoprotein-associated particles, thereby reducing proteomic interference from high-abundance plasma components. In plasma-derived EV proteomics, reduction of abundant plasma proteins and lipoproteins, including ApoA1- and ApoB-associated particles, is methodologically critical, as these components may dominate MS spectra and obscure low-abundance vesicular cargo. Therefore, prioritising contaminant reduction over maximal vesicle yield was considered essential for improving analytical specificity and proteomic depth in the present biomarker-oriented study.

Importantly, a recent comparative study demonstrated that while both 35 nm and 70 nm series effectively isolate small EVs from plasma, the 70 nm configuration yields a higher relative abundance of vesicle-associated proteins in EV-enriched fractions, supporting its suitability for downstream proteomic analyses [137]. Although this configuration may reduce overall particle recovery, the improved enrichment of vesicle-derived proteins was considered advantageous for quantitative MS-based workflows. Thus, the selection of qEV 70 nm columns represented a deliberate methodological compromise favouring analytical specificity and proteomic depth over maximal vesicle yield, consistent with the biomarker-oriented design of the present study.

Integration of SEC-based EV isolation with downstream MS-based proteomic analysis has been supported by methodological studies, including Vanderboom et al.[138] demonstrating improved separation of vesicle-enriched fractions from plasma protein contaminants compared with ultracentrifugation-based approaches. Such optimisation is particularly important in studies aiming to detect subtle disease-associated alterations within the EV proteome, as is the case in endometriosis, where contamination by high-abundance circulating proteins may distort quantitative analyses and reduce analytical sensitivity.

In accordance with the MISEV recommendations[76], EV identity and purity were validated using complementary orthogonal approaches in both plasma- and PF-derived EV preparations. Both scatter-mode NTA and F-NTA were applied to assess particle size distribution and concentration, as well as membrane integrity (CMDR staining) and expression of canonical tetraspanins. These findings were further confirmed by Western blot analysis demonstrating enrichment of EV-associated proteins (CD9, CD81, EDH4, Tsg101) and absence of the endoplasmic reticulum marker calnexin, excluding significant cellular

contamination. Cryo-EM microscopy provided ultrastructural confirmation of intact, membrane-bound vesicles displaying a characteristic lipid bilayer.

This multi-layered validation framework gave us the confidence that subsequent proteomic alterations reflect genuine changes in vesicle-associated cargo rather than technical artefacts, protein aggregates, or non-vesicular material. Such methodological rigor is particularly important in endometriosis, where anticipated disease-associated signatures are likely to be subtle and predominantly quantitative rather than driven by overt morphological differences.

Interestingly, quantitative analysis revealed detectable systemic alterations in plasma-derived EVs in endometriosis. We noticed an increased abundance of membrane-positive vesicles detected in fluorescence-mode NTA and shifts in scatter-defined size distribution towards smaller vesicles. Notably, total particle concentration assessed in scatter mode did not differ significantly between groups, indicating that the observed differences were specific to membrane-enclosed vesicular structures rather than to the overall nanoscale particle population. Given that scatter-mode NTA does not discriminate between vesicular and non-vesicular particles of similar size, the selective increase in CMDR-positive vesicles strengthens the interpretation that systemic changes in endometriosis reflect genuine alterations in circulating EV biology rather than nonspecific fluctuations in particle background.

In contrast, PF-derived EVs did not exhibit significant global differences in particle number or size between endometriosis patients and controls. Quantitative NTA of PF samples demonstrated comparable particle concentrations and size distributions across groups (data not shown). This divergence suggests that systemic manifestations of endometriosis may be captured at the level of vesicle abundance and biophysical characteristics, whereas local peritoneal pathology may be predominantly encoded in qualitative alterations of vesicular cargo composition rather than in total particle count or size. Such compartmentalisation of inflammatory and immune responses between the peritoneal cavity and systemic circulation has been described in endometriosis[8, 23] and other chronic inflammatory conditions [139].

These observations have important implications for circulating EV-based biomarker development. They indicate that systemic EV measurements may detect disease-associated alterations even in the absence of measurable quantitative changes within the local peritoneal compartment. Overall, these results are in line with reports that have are increasingly recognised EVs as mediators of intercellular communication in immune regulation and

chronic inflammatory processes, and implicated EV dysregulation in endometriosis pathophysiology[89].

Accordingly, while quantitative EV analysis provided evidence for systemic vesicle alterations, it remained insufficient to fully characterise the molecular mechanisms underlying endometriosis-associated changes. Therefore, high-resolution TMT-based quantitative proteomic profiling was performed to identify disease-associated molecular signatures and to differentiate between local peritoneal processes and their systemic manifestations.

In parallel to the investigated patient and control samples, we have incorporated into the TMT multiplex design reference channels containing pooled cell proteomes and cell-derived EVs from endometriosis-derived and healthy decidualized endometrial stromal cell lines, thereby reducing the impact of plasma protein dominance and facilitating more confident interpretation of vesicle-associated signals. The resulting proteomic landscape revealed compartment-specific patterns, providing a functional extension of the quantitative vesicle analysis and establishing the basis for subsequent biological interpretation. we next examined whether these compartment-specific datasets translate into biologically meaningful disease-associated signatures.

To support the interpretation of EV-derived proteomic data, cell line-derived reference proteomes and EVs were incorporated into the experimental design. The selection of the 12Z endometriotic epithelial cell line and the MDAH-2774 ovarian endometrioid adenocarcinoma cell line was guided by their relevance to endometriosis and its known association with ovarian cancer (OvCa). Endometriosis is characterized by invasive and angiogenic properties that share mechanistic overlap with tumour-related pathways, while ovarian endometrioid carcinoma represents a clinically and biologically linked condition. The inclusion of both cell models therefore enabled a broader contextual interpretation of EV-associated proteins, capturing molecular features related to both benign and transformation-associated processes.

5.2. EVs as potential immunomodulatory biomarkers reflecting disease progression in endometriosis

Building upon the established methodological framework, high-resolution TMT-based quantitative proteomic analysis of PF- and plasma-derived EVs revealed distinct compartment-specific molecular signatures in advanced endometriosis.

The global proteomic analysis identified and quantified 2,600 proteins in plasma-derived EVs and 3,571 proteins in PF-derived EVs from endometriosis patients and controls, with 2,304 proteins shared between both biofluids. Such a relatively high number of detected

proteins is notable for EV-based analyses, particularly in plasma-derived vesicles, where the high dynamic range of circulating proteins and co-isolated abundant plasma components frequently limit identification sensitivity. For example, Karimi et al. identified 1,187 proteins in plasma-derived EVs after extensive lipoprotein depletion[131], while other EV proteomic studies of PF reported considerably smaller protein repertoires, often below 200 identified proteins in PF-derived exosomes[140]. Importantly, this level of coverage compares favourably with previously published EV proteomics datasets, in which identification breadth is often constrained by the overall complexity of biological samples and the presence of highly abundant proteins that may hinder the detection of less abundant EV-associated components.
The extensive depth of proteomic profiling achieved in the present study likely reflects the combined effects of optimized SEC-based EV isolation, TMT-based multiplexed quantitative proteomics, and the incorporation of biologically informative reference channels, including (CD-EVs), within the experimental design. This strategy expanded the peptide identification space while preserving unbiased statistical comparisons between the clinical cohorts.

By integrating EV profiles from both anatomical compartments within the same individuals, we could directly compare local peritoneal and systemic molecular alterations, addressing a major limitation of previous biomarker studies that typically focused on a single biofluid.

PCA and hierarchical clustering of EV-related proteins demonstrated more robust separation between endometriosis and control groups in PF than in plasma. Consistently, hierarchical clustering showed tight intra-group clustering and distinct group separation in PF-derived EVs, whereas plasma profiles exhibited greater dispersion and partial intergroup overlap. These findings indicate that PF-derived EVs capture localized molecular perturbations with higher resolution, reflecting the immediate inflammatory and stromal microenvironment of the peritoneal cavity. In contrast, plasma-derived EVs appear to represent a more heterogeneous systemic signal, likely influenced by multiple tissue sources of the EVs and systemic immune modulation. Notably, the single stage IV case exhibited a distinct proteomic profile in both plasma- and PF-derived EVs. Although this observation is based on a single case and therefore requires validation in larger cohorts, it may suggest that progressive lesion complexity and advanced pathological remodelling are accompanied by measurable alterations in EV cargo composition. This raises the possibility that EV proteomic patterns could reflect not only disease presence but also clinical severity or phenotype.

Collectively, these findings support the concept that EVs act as compartment-sensitive reporters of disease activity in endometriosis. PF-derived EVs appear particularly informative

for capturing localized pathological processes, whereas plasma-derived EVs may hold translational potential for minimally invasive systemic monitoring. This compartmental distinction provides a conceptual framework for interpreting subsequent analyses of DEPs aimed at the identification of endometriosis-relevant signalling pathways and processes. Like in immune regulation, oxidative stress, ECM remodelling, and cellular adhesion.

5.3. Proteomic profiling of PF-derived EVs: insight into the local disease environment

Compared to plasma-derived EVs, PF-derived EVs exhibit a more distinct and sensitive proteomic signature, characterized by stronger group separation, more defined clustering patterns, and a broader repertoire of differentially expressed proteins. This enhanced resolution suggests that PF EVs provide superior sensitivity for detecting disease-specific molecular alterations within the local microenvironment. Given the stronger discriminatory capacity observed in PF-derived EVs, we first focused on the local peritoneal compartment to dissect the biological pathways underlying lesion-associated molecular alterations. Proteomic profiling of PF-derived EVs revealed a distinct set of DEPs involved in key biological processes underlying endometriosis progression, including epigenetic regulation, oxidative stress and iron homeostasis, immune modulation, adhesion and migration and ECM remodelling. These shifts may reflect the dynamic nature of lesion progression, including changes in proliferation, differentiation, and microenvironmental adaptation. To integrate these findings into a coherent mechanistic framework, a conceptual model of PF-derived EV-associated processes within the peritoneal microenvironment is presented (Figure 33).

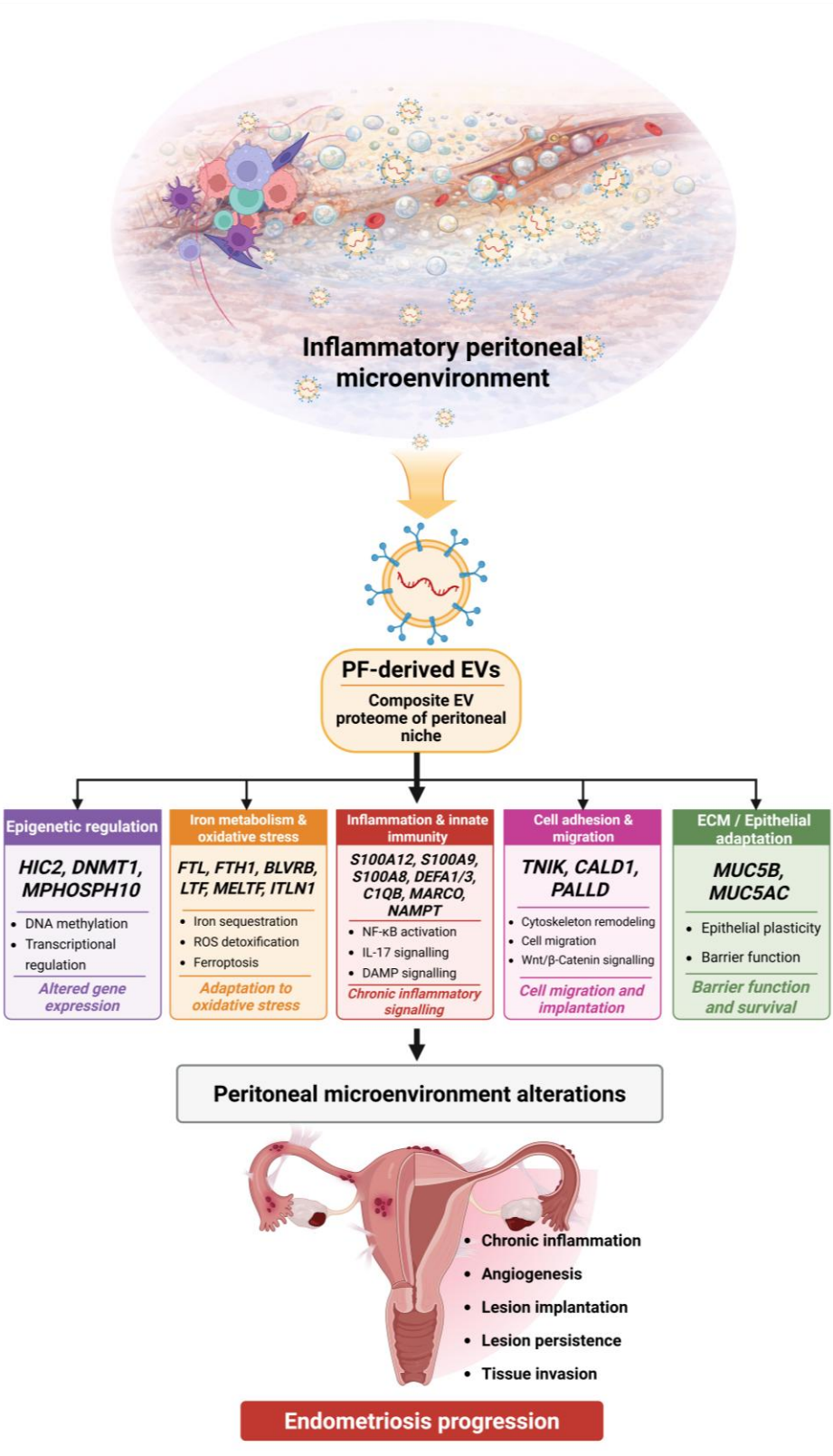

Figure 33. PF-derived EVs reflect integrated pathogenic mechanisms shaping the peritoneal microenvironment in endometriosis. EV-associated proteins identified in PF converge on key biological processes, including epigenetic regulation, oxidative stress and iron metabolism, inflammatory and innate immune signalling, cell adhesion and migration, and epithelial/ECM remodelling. These pathways collectively contribute to the establishment of a permissive peritoneal niche characterised by chronic inflammation, enhanced cellular plasticity, and increased invasive potential. Selected representative proteins identified in the proteomic dataset are shown for each functional category. Together, these findings support the concept that PF-derived EVs act as integrators of local disease-associated signals and may actively contribute to lesion development and persistence.

In the following section, we discuss the most strongly upregulated proteins and examine their potential roles in driving key pathogenic mechanisms in endometriosis. One of the most strongly upregulated proteins was HIC2 (FC = 16.57), a transcriptional repressor with epigenetic regulatory potential. The high abundance of HIC2 may suggest an attempt to counterbalance excessive cellular proliferation or indicate stromal adaptation. This would be consistent with prior evidence implicating HIC family transcription factors in fibroblast transformation and repression of Wnt signalling[141, 142]. However, a direct functional role for HIC2 in endometriosis has not been established. To further explore the relevance of this signal, we examined HIC2 tissue-level expression in the Turku EndometDB resource[143], which integrates datasets from eutopic endometrium, peritoneum, and various endometriosis lesion types (peritoneal, deep infiltrating, and ovarian), allowing comparison across different anatomical sites. Although HIC2 was not among the top differentially expressed genes, its expression was consistently detectable and showed modest elevation in deep infiltrating and ovarian endometriosis lesions compared to control peritoneum and eutopic endometrium. This pattern supports the possibility that HIC2-positive stromal or fibroblast populations may contribute to its EV-based signal, and that its presence in EVs may result from selective packaging mechanisms rather than direct correlation with bulk tissue abundance. Notably, HIC2 belongs to the HIC family of transcriptional repressors and is closely related to HIC1, a well-known epigenetic regulator involved in chromatin remodelling and transcriptional control. Within this regulatory context, sirtuin 1 (SIRT1), a NAD^+-dependent deacetylase involved in epigenetic regulation, inflammation, and cellular stress responses[144], has been repeatedly reported to be upregulated in endometriosis and linked to progesterone resistance and epithelial-to-mesenchymal transition, processes central to lesion persistence and progression[145, 146]. Against this background, elevated HIC2 levels may represent a context-dependent regulatory response within stromal compartments that modulates SIRT1-associated pathways rather than acting as a primary disease driver. Although the functional directionality of HIC2-SIRT1 signalling in endometriotic tissue remains unresolved, this association provides a biologically plausible link between EV-associated HIC2 and known epigenetic and inflammatory mechanisms in endometriosis[147].

MPHOSPH10 , the second most upregulated protein in our dataset (FC = 4.59), is a mitotic phosphoprotein involved in ribosomal RNA processing and nucleolar organization[148]. Although its specific role in endometriosis also remains unclear, transcriptomic analyses of uterine fluid and endometrial samples have detected MPHOSPH10 expression in women with advanced-stage disease[149], suggesting a possible association with disease severity or

proliferative activity. Notably, MPHOSPH10 is a core component of the U3 small nucleolar ribonucleoprotein (snoRNP) complex and is tightly regulated during mitotic (M)-phase progression[150], suggesting a potential link to cellular proliferation dynamics within endometriotic lesions. Its high abundance in EVs may reflect the proliferative or transcriptionally active state of the source cells, rather than lesion specificity. Moreover, its known correlation with patient survival in several cancer types, including OvCa[151], underscores its relevance as a proliferation-related marker that may warrant further investigation in endometriosis-associated neoplasia or aggressive phenotypes.

Among other top upregulated proteins in PF-derived EVs, DNMT1 stands out as a key regulator of DNA methylation and epigenetic stability. Notably, DNMT1, along with DNA methyltransferases 3A and 3B (DNMT3A, DNMT3B), has been shown to be significantly overexpressed in epithelial cells from ectopic endometrial lesions compared to both eutopic and healthy endometrium, suggesting widespread methylation disturbances as a hallmark of endometriosis152. Crucially, estrogen upregulates DNMT1 expression in eutopic stromal cells, promoting hypermethylation and silencing of the tumour suppressor gene RUNX3, a mechanism directly implicated in the malignant transformation of ovarian endometriosis[153]. onsistently, STRING analysis clustered DNMT1 with other chromatin and transcriptional regulators, reinforcing its central role in epigenetic reprogramming. In light of our previous review, which highlights the role of immunosuppressive EVs as a linking factor in the progression of both endometriotic and tumour lesions115, the presence of DNMT1 in EVs may reflect an active contribution to epigenetic reprogramming of the local microenvironment. This, in turn, could facilitate the emergence of more aggressive, transformation-prone phenotypes, underscoring the importance of EV-associated epigenetic regulators in shaping endometriosis pathology.

Dysregulated iron homeostasis and the associated oxidative stress are additional contributors to lesion progression in endometriosis. In our dataset, we observed increased abundance of several iron-handling and detoxification proteins, including ferritin light chain (FTL), ferritin heavy chain 1 (FTH1), lactotransferrin (LTF), melanotransferrin (MELTF), biliverdin reductase B (BLVRB), and intelectin 1 (ITLN1), which have been previously linked to endometriosis via altered iron metabolism, chronic inflammation, and ferroptosis.

GO analysis highlighted pathways such as “sequestering of metal ion” and “iron ion transport”, with “intracellular ferritin complex”. STRING network clustering grouped these proteins in a module annotated as “iron metabolism and oxidative microenvironment”, and

Reactome/KEGG enrichment analyses pointed to “metal sequestration by antimicrobial peptides” and “ferroptosis,” supporting their role in iron-driven oxidative stress.

FTL and BLVRB were the most strongly upregulated DEPs, indicating key roles in adaptation to iron overload. FTL, a ferritin core protein, was upregulated in PF-derived EVs (FC = 2.26) and detectable in plasma (FC = 1.21). It prevents Fenton chemistry and has been found elevated in endometriotic lesions, contributing to ferroptosis and tissue damage[154]. Iron exposure upregulates FTL in endometrial stromal cells via divalent metal transporter 1 (DMT1)[155], and in peritoneal macrophages it correlates with impaired phagocytic capacity and enhanced secretion of IL-8 and vascular endothelial growth factor A (VEGFA), promoting angiogenesis and lesion survival[156]. BLVRB, an enzyme involved in heme catabolism and elevated in ectopic compared to eutopic endometrium[157]. It converts biliverdin to bilirubin, counteracting reactive oxygen species (ROS) accumulation and supporting ectopic endometrial cells survival within the iron-rich, pro-oxidant peritoneal environment. Intelectin 1 (ITLN1) appears to modulate local responses to heme breakdown and oxidative stress[158] and has also been implicated in OvCa, where it suppresses lactoferrin-driven invasiveness via matrix metalloproteinase 1 (MMP1) downregulation and metabolic reprogramming[159]. Although not among the top upregulated EV proteins in our dataset, FTH1 deserves special mention due to its emerging relevance in both endometriosis and OvCa. Overexpressed FTH1 supports epithelial-mesenchymal transition and protects cells from iron-induced oxidative damage through intracellular iron storage and ROS detoxification in endometriotic lesions [160, 161]. It has been implicated in the progression of endometriosis-associated ovarian clear cell carcinoma (OCCC)[162].

Beyond iron detoxification, metabolic adaptations may help ectopic cells survive in the oxidative and inflammatory environment of endometriotic lesions. We detected elevated expression of solute carrier family 2 member 3 (SLC2A3), also known as glucose transporter type 3 (GLUT3), a high-affinity glucose transporter known to be overexpressed in eutopic endometrium from women with endometriosis and present in ectopic lesions[163]. This suggests enhanced glucose uptake and metabolic flexibility under hypoxia or oxidative stress, paralleling adaptations seen in cancer-like phenotypes.

Iron-driven oxidative stress sustains chronic inflammation and pathological angiogenesis in endometriotic lesions. In line with this concept, we observed EV-associated upregulation of multiple proteins involved in innate immunity and vascular remodelling, including S100A12, S100A9, S100A8, defensin alpha 1/3 (DEFA1/3), complement component 1q subcomponent subunit B (C1QB), macrophage receptor with collagenous

structure (MARCO), nicotinamide phosphoribosyltransferase (NAMPT), protein arginine methyltransferase 1 (PRMT1), and heat shock protein family A members 6 and 7 (HSPA6, HSPA7), all of which are known mediators of inflammatory, immune, or angiogenic pathways relevant to endometriosis. Enrichment analyses supported this inflammatory signature, identifying GO terms such as “chronic innate immune response” and KEGG pathways including “IL-17 signalling” and “necroptosis.” S100A12, S100A9, and S100A8—calcium-binding alarmins acting as endogenous danger signals (damage-associated molecular patterns, DAMPs) and released by activated neutrophils—were notably enriched in both PF- and plasma-derived EVs. S100A12 showed the strongest increase (FC = 2.86 in PF, 2.08 in plasma), followed by S100A9 (FC = 2.01 and 1.59) and S100A8 (FC = 1.71 and 1.29). Acting through RAGE and toll-like receptor 4 (TLR4), these DAMPs drive nuclear factor kappa B (NF-κB) activation, cytokine release, and endothelial activation, promoting persistent inflammation. Elevated S100A12 expression has been reported ectopic endometrium[164]. Its expression is induced by lipopolysaccharide (LPS) and downregulated by atorvastatin[165], and it is already patented as a potential biomarker and therapeutic target for endometriosis (WO2010029497A1)[166]. S100A8/A9 form proinflammatory complexes that induce MMPs and contribute to menstrual tissue breakdown. Their sequential, progesterone-regulated expression in stromal cells[167] and increased levels in menstrual blood of endometriosis patients[168] support their role in cyclical remodelling. Beyond their role in cyclical tissue breakdown, S100A8/A9 also drive fibrosis through the receptor for advanced glycation end products (RAGE)–Janus kinase 2 (JAK2)–signal transducer and activator of transcription 3 (STAT3) signalling pathway, contributing to intrauterine adhesions (IUA), while menstrual blood-derived stromal cells (MenSC) transplantation reverses this effect[169]. Systemically, elevated plasma S100A9 was associated with increased endometriosis risk in the Nurses’ Health Study II28, supporting its utility as an early systemic biomarker[170]. Single-cell RNA-seq further identified S100A9^{+} macrophages in eutopic endometrium, linked to pro-angiogenic activity and lesion growth, which were suppressed by Tasquinimod in a mouse model[171]. Altogether, S100A8, S100A9, and S100A12 are robust inflammatory markers with strong diagnostic and therapeutic potential in endometriosis.

DEFA1 and DEFA3 encode α-defensins (HNP1/3), antimicrobial peptides with immunomodulatory and pro-angiogenic properties. Elevated in PF of endometriosis patients, they correlate with neutrophil counts and IL-8, suggesting active inflammatory roles[172]. Recent studies revealed pro-angiogenic neutrophils in menstrual effluent and murine models, promoting lesion formation via neutrophil extracellular traps (NETs), cell adhesion support,

and angiogenic stimulation[173]. Their EV enrichment reflects this neutrophil activity, indicating that α-defensins may be both biomarkers and mediators of early lesion development.

The same inflammatory milieu also promotes cytoskeletal and adhesive remodelling, crucial for lesion implantation and persistence. Several PF-EV proteins involved in these pathways formed STRING and KEGG clusters annotated as “ECM interaction” and “cell adhesion”. Among them, TRAF2 and NCK-interacting kinase (TNIK) - a redox-sensitive kinase linking inflammation to actin remodelling via ezrin–radixin–moesin (ERM) phosphorylation- was strongly upregulated. It activates Wnt/β-catenin signalling and transcription factor 4 (TCF4)-dependent transcription[174, 175]. Although downregulated in eutopic endometrium[176], its EV enrichment suggests post-transcriptional or selective release during lesion formation.

CALD1, another actin-binding protein, regulates actomyosin contractility and cell motility, modulating cell shape, stiffness, and migration. Its reduced expression in eutopic endometrium correlates with increased cell motility under inflammatory conditions[177, 178]. We observed CALD1 downregulation in PF-EVs (FC= -1.22, FDR < 0.05), and a similar but non-significant trend in plasma EVs (FC= -1.10), suggesting shared but compartment-specific regulation and suggesting a role in early lesion migration. Interestingly, CALD1 is elevated in mature ectopic lesions, indicating a dual role-supporting initial invasion and later cytoskeletal stabilization and anchoring within the ectopic tissue microenvironment[177, 179]. CALD1 also clustered with PALLD, a scaffold protein involved in cytoskeletal stability and migration. PALLD is consistently downregulated in endometriosis[180, 181], pointing to impaired anchoring but enhanced plasticity in ectopic cells. This CALD1-PALLD axis exemplifies how cytoskeletal remodelling actively drives lesion progression, linking inflammation to structural adaptation.

Epithelial adaptations further support ectopic survival. MUC5B, a gel-forming mucin, is upregulated in PF-EVs (FC = 2.03) and modestly in plasma (FC = 1.39). It enhances barrier function and apoptosis resistance under inflammatory stress. It enhances barrier function and apoptosis resistance under inflammatory stress. A distinct MUC5B^{+}/ trefoil factor 3 (TFF3)$^{-}$ epithelial population has been identified in lesions[182], and MUC5B is also upregulated in endometrial adenocarcinoma[183], suggesting broader roles in survival and immune evasion. In contrast, MUC5AC, common in ovarian and cervical cancers,[184, 185] is mostly absent in endometriosis tissues[186]. Its EV presence may indicate atypical glandular features or metaplastic changes, not overt malignancy.

Overall, PF-derived EVs appear to actively modulate the peritoneal niche in endometriosis. Their cargo supports inflammation, migration, adhesion, and survival, and may contribute to malignant transformation in endometriosis-associated ovarian cancer (EAOC). These findings highlight the potential of EVs as both biomarkers and therapeutic targets. While PF-derived EVs primarily reflect localized inflammatory and stromal remodelling, it remained essential to determine how these alterations are mirrored or modified within the systemic circulation.

5.3. Proteomic profiling of plasma-derived EVs: Insight into systemic responses in endometriosis

Proteomic profiling of plasma-derived EVs revealed a distinct but partially overlapping set of DEPs compared to PF-derived EVs. Though fewer in number and with generally lower fold changes, several plasma EV proteins were significantly upregulated, reflecting systemic processes relevant to endometriosis progression. Notably, many were linked to intracellular signalling and vesicle transport - key processes for proliferation, adhesion, and immune modulation.

PIK3C2A was the most strongly upregulated plasma-EV protein (FC = 7.37). It encodes a class II phosphoinositide 3-kinase involved in PI3K/AKT signalling, a pathway central to cell proliferation, survival, and adhesion. It was previously reported as differentially expressed in eutopic and ectopic endometrium[187, 188], PIK3C2A may drive apoptosis resistance and lesion growth. Its presence in plasma EVs suggests a potential systemic marker of active or disseminating disease. STRING analysis linked PIK3C2A to CLTCL1 (FC = 2.98), forming a cluster enriched in endocytosis and growth signalling. CLTCL1 encodes a clathrin-like protein involved in receptor recycling and intracellular trafficking[189]. Though not yet studied in endometriosis, CLTCL1 is a prognostic marker in endometrial carcinoma[189] and part of a programmed cell death signature in OvCa[190].

SDC4 (FC = 3.17) also stood out in plasma EVs. A transmembrane proteoglycan, SDC4 facilitates cell-ECM adhesion, motility, and cytoskeletal remodelling. It's upregulated in stromal cells from deep endometriosis[191] and linked to telomerase-responsive gene networks[192]. Detected in both plasma and PF EVs, SDC4 may support ectopic implantation and serve as a promising circulating biomarker. Within the same network, PODXL (FC = 3.17), a sialomucin regulating cell-cell and ECM interactions, was also enriched. Identified in endometriosis transcriptomic datasets[193], PODXL may enhance migratory

capacity and peripheral lesion formation. Though not directly interacting with SDC4, both map to a STRING cluster centred on EZR (FC = 1.56), a cytoskeletal linker that promotes motility via RhoA/ROCK activation[194, 195]. Elevated EZR and phospho-EZR have been reported in eutopic and ectopic tissues[196, 197], supporting its role in invasion. Together, the enrichment of SDC4, PODXL, and EZR in plasma EVs highlights a systemic cytoskeletal-adhesive axis that mirrors key mechanisms of ectopic lesion establishment and spread. Their coordinated upregulation suggests EVs may not only reflect but also mediate systemic disease progression in endometriosis. Altogether, these findings demonstrate that EV proteins dysregulated in endometriosis are not functionally isolated but instead form interconnected modules, reflecting coordinated mechanisms involved in immune dysregulation, oxidative stress, and cell–cell communication central to disease pathophysiology.

Detection of DEAD-box RNA helicases in plasma-derived EVs suggests systemic activation of RNA processing in endometriosis. DDX47 and DDX31 were among the most enriched (FC = 2.10 and 2.09), with DDX47 reaching FDR significance. Both are involved in ribosome biogenesis, mRNA transport, and translational regulation, and clustered together in a STRING module annotated as "RNA processing and regulatory machinery." DDX47 is expressed in endometrial tissues (Human Protein Atlas)[198], while DDX31has been linked to endometriosis risk via large-scale genome-wide association studies (GWAS) at the ABO/9q34.2 locus[199], suggesting their roles in RNA-mediated adaptation to inflammation or cellular stress. In contrast, TOP2B, a type II topoisomerase involved in chromatin remodelling, was found to be downregulated (FC = -1.59). It facilitates estrogen-regulated transcription by relieving DNA torsional stress[200]. Although primarily described in estrogen-sensitive cancers, similar chromatin-regulatory mechanisms likely operate in other estrogen-responsive tissues, including the endometrium. Reduced TOP2B may limit transcriptional responses to estrogen, contributing to hormone resistance in endometriotic lesions and impaired gene regulation under chronic hormonal stimulation. Given the well-established role of estrogen signalling in endometriosis pathogenesis, reduced TOP2B expression may contribute to impaired hormone-driven gene regulation and resistance to endocrine therapies, pointing to an emerging regulatory role of chromatin-modifying enzymes that requires further investigation.

As in PF, plasma-EVs also contained representants of mucins like MUC1 and MUC16. MUC16 (a transmembrane glycoprotein better known as CA125, FC = 2.09), a well-known marker for endometriosis and OvCa, as highlighted in a recent review[201]. It, is elevated especially in advanced-stage disease, where levels frequently exceed 35 U/ml[202] and

correlates with lesion burden[203], infertility[204], and deep infiltrating endometriosis (DIE), particularly in the presence of endometriomas[205]. While not specific, its dynamic changes across the cycle improve diagnostic value[206].A combination of CA125 with CA19-9, HE4, and epididymal protein achieved an AUC of 0.900 and 85.4% sensitivity[207]. CA125 is also negatively correlated with anti-Müllerian hormone (AMH), useful for monitoring treatment response[208]. Beyond diagnosis, CA125 is useful for monitoring treatment response-declines after therapy reflect improvement, whereas recurrent elevation may signal relapse, especially in ovarian endometriosis[209, 210]. Although rare, malignant transformation of endometriosis increases the risk of clear cell and endometrioid OvCa, where a sharp CA125 rise should raise suspicion[211]. In such contexts, its combination with human epididymis protein 4 (HE4) supports early cancer detection[212]. Beyond diagnostics, CA125 is being explored as a therapeutic target (e.g., oregovomab)[213]. Functionally, MUC16 may facilitate immune evasion and mesothelial clearance, as described in OvCa[214].

Mesothelin (MSLN), another enriched protein, forms a STRING cluster with MUC16 and MUC1 involved in “mesothelial interaction and adhesion. MSLN is expressed by mesothelial cells and is associated with adhesion, migration, and angiogenesis. Though not directly implicated in mesothelial-to-mesenchymal transition (MMT), its EV enrichment may reflect mesothelial activation and peritoneal fibrosis driven by TGF-β signalling and platelet activation[215]. In cancer models, MSLN has been shown to promote angiogenesis, immunosuppression, and cell migration[216, 217], mechanisms that may be analogous to those observed in endometriosis. Interestingly, the CA125–mesothelin complex is less common in endometriosis than in OvCa, supporting its differential diagnostic potential[218]. Serum MSLN is elevated in endometriosis and decreases with probiotics, suggesting its role as a marker of inflammation and peritoneal injury and inflammation[219].

Accompanying local and systemic immunosuppression appear to play a pivotal role in the persistence and progression of ectopic lesions and in this context FGL2, significantly enriched in plasma EVs ($FC > 1.5$, $FDR < 0.05$), emerged as a key immunomodulatory protein. Secreted by Tregs, soluble FGL2 induces macrophage polarization toward an M2 phenotype via SHP2-ERK1/2-STAT3, suppressing NF-κB and promoting immune tolerance[220]. FGL2 was also identified among efferocytosis-related genes involved in inflammation and immune escape[221]. Although its diagnostic accuracy is limited, its consistent EV presence highlights its relevance as a systemic immunoregulatory mediator and a potential therapeutic target.

Together, these findings indicate that plasma-derived EVs from endometriosis patients harbour a distinct protein signature enriched in biological processes related to immune response and tissue remodelling. To further summarise the systemic processes reflected by plasma-derived EVs, an integrated overview of the major functional pathways is presented (Figure 34).

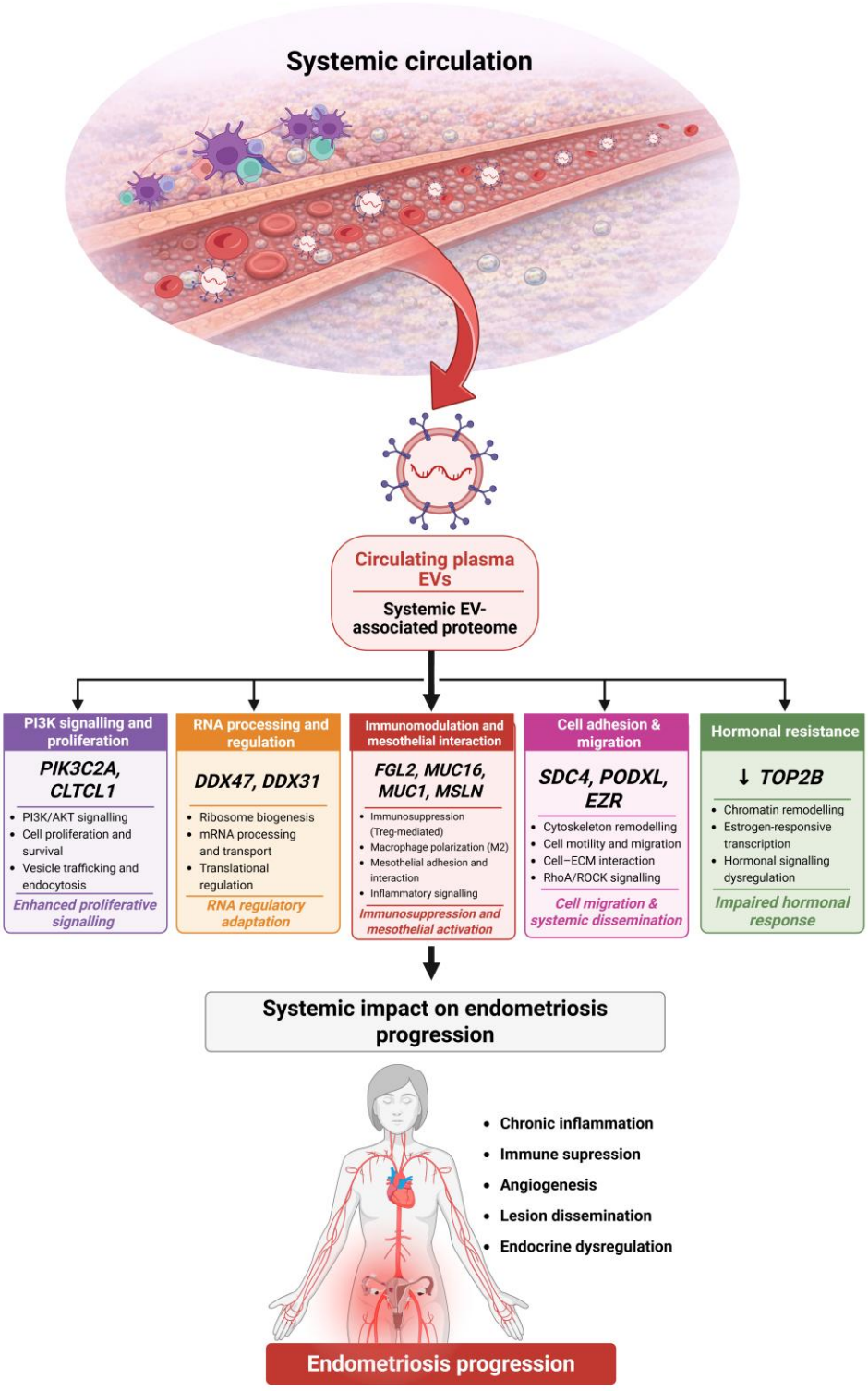

Figure 34. Circulating plasma-derived EVs reflect systemic processes associated with endometriosis progression. EV-associated proteins identified in plasma converge on key biological pathways, including proliferative signalling, RNA processing and translational regulation, immune modulation, cell adhesion and migration, and hormonal dysregulation. These processes collectively reflect a systemic response characterised by chronic inflammation, altered immune activity, and increased cellular plasticity. Selected representative proteins identified in the proteomic dataset are shown for each functional category. Together, these findings support the concept that circulating EVs extend beyond the local peritoneal environment and represent a systemic layer of disease-associated signalling, reinforcing their potential as minimally invasive biomarkers in endometriosis.

This supports the concept that circulating EVs reflect systemic disease processes and may serve as accessible biomarkers for endometriosis detection and monitoring. Overall, despite showing lower discriminatory power than PF EVs, plasma EVs offer accessible, minimally invasive biomarkers reflecting systemic mechanisms of endometriosis. Their proteomic profile aligns with PF EVs in key pathways-immune modulation, inflammation, adhesion, signalling, and cytoskeletal remodelling-underscoring their translational value in diagnostics and mechanistic research.

5.3. Shared EV-associated proteins reflect core pathomechanisms of endometriosis across local and systemic compartments and reveal translational biomarker potential

Beyond compartment-specific alterations, a substantial subset of proteins was detected in both PF and plasma-derived EVs, suggesting the existence of core disease-associated signatures that transcend anatomical boundaries. Given the anatomical proximity of the peritoneal cavity to the vascular system and its extensive surface area, molecular alterations originating locally at the site of endometriotic lesions may also be detectable in EVs circulating in the bloodstream. The concurrent presence of DEPs both in PF and plasma, clustering into functionally coherent modules representing mechanisms central to the progression of endometriosis, suggests a viable route toward minimally invasive biomarker discovery. To integrate these observations, a conceptual framework illustrating shared EV-associated mechanisms across local and systemic compartments is presented (Figure 35).

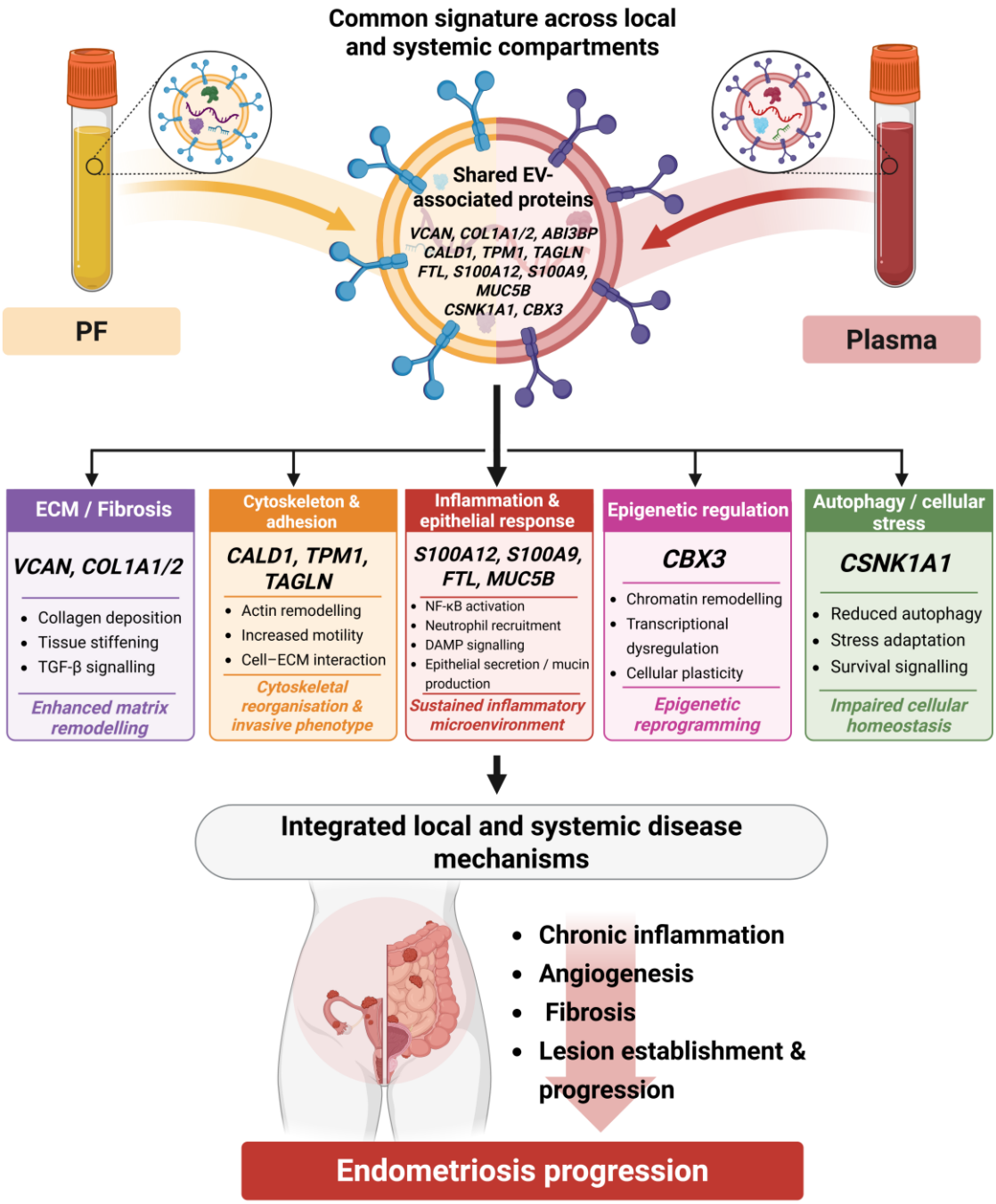

Figure 35. Shared EV-associated proteins define core molecular mechanisms linking local and systemic compartments in endometriosis. A subset of EV-associated proteins was consistently detected in both PF and plasma, indicating the presence of a common molecular signature that transcends anatomical compartments. These shared proteins converge on key biological processes, including ECM remodelling, cytoskeletal reorganisation, sustained inflammatory signalling, epigenetic regulation, and impaired cellular homeostasis. Selected representative proteins identified in the proteomic dataset are shown for each functional category. The coordinated regulation of these pathways suggests that EVs mediate the integration of local lesion-associated signals with systemic responses, thereby contributing to a permissive environment for lesion establishment and progression. In this context, shared EV-associated proteins may represent a mechanistic link between peritoneal pathology and circulating biomarkers, supporting their potential utility in minimally invasive disease detection and monitoring.

A subset of proteins showed higher expression in PF-derived EVs (e.g., FTL, S100 family, MUC5B), suggesting local production and lesion-specific involvement, while others like SDC4 and FGL2 were more enriched in plasma-derived EVs, indicating systemic effects. These proteins have been discussed earlier according to their primary site of expression. Despite variations, most DEPs showed consistent directional changes and fold changes nearing or exceeding 1.5 in both fluids, supporting active molecular exchange between the peritoneal and systemic compartments. This reinforces the potential of circulating EVs as carriers of localized disease signals, contributing to non-invasive biomarker development for endometriosis. Additionally, proteins with moderate but coherent fold changes (FC ≈ 1.2-1.5) across both fluids may still hold value as part of multifactorial molecular profiles. Though individually limited as biomarkers, they could enhance multi-omics panels. Targeted validation using parallel reaction monitoring (PRM) or multiple reaction monitoring (MRM) could establish their clinical relevance.

Proteins such as VCAN, COL1A1/2, and ABI3BP, linked to ECM remodelling and tissue stiffness, were present in both plasma and PF-EVs, indicating stromal activation. VCAN showed stronger enrichment in PF EVs (FC = 1.56 vs. 1.16 in plasma) and was functionally connected to SDC4. Versican, a key chondroitin sulfate proteoglycan of the ECM, promotes endometrial cell adhesion/invasion and especially the V1 isoform is upregulated in peritoneal endometriosis[222]. Functional assays showed that VCAN V1 promotes both adhesion and invasion of endometrial stromal cells (ESCs) into mesothelial cells and through the ECM, indicating a direct role in peritoneal colonization. These findings suggest that peritoneal versican actively contributes to the early phases of lesion establishment. VCAN expression is TGF-β1-inducible but not directly modulated by PF, suggesting intrinsic mesothelial dysregulation. Transcriptomic data show increased VCAN in advanced-stage eutopic endometrium, particularly during the secretory phase of the menstrual cycle, suggesting hormonal regulation and a role in ECM remodelling and cell migration[157, 180]. Together, these data suggest that altered VCAN signalling-whether through local tissue overexpression or systemic regulatory inhibition-may contribute to a permissive environment for ectopic lesion development, offering potential diagnostic and therapeutic targets.

COL1A1/2, encoding type I collagen, were moderately upregulated in both fluids (COL1A1: FC = 1.25 PF, 1.19 plasma; COL1A2: FC = 1.23 PF, 1.43 plasma). Their presence in EVs suggests active stromal reprogramming and fibrotic processes, characteristic of long-standing and deeply infiltrating forms of the disease. Their STRING clustering and literature data link them to fibrosis and impaired decidualization in endometriosis. Elevated levels of

COL1A1 and COL1A2 mRNA have been associated with defective decidualization and impaired endometrial receptivity in endometriosis-related infertility, potentially disrupting progesterone-mediated endometrial transformation[223]. COL1A1/A2 promoted directional migration, invasion, and ECM remodelling by both stromal and epithelial endometrial cells[224]. Collectively, these findings implicate type I collagen not only in the fibrotic phenotype of advanced endometriosis, but also in the disruption of physiological endometrial function.

Cytoskeleton-associated proteins (CALD1, TPM1, TAGLN) formed a functional cluster of cytoskeletal regulation, cellular plasticity, and cell-ECM interactions, whose coordinated activity may play a crucial role in the initiation of adhesion and migration of endometrial cells on the peritoneal surface. TPM1, a protein involved in actin cytoskeleton regulation, muscle contraction, cell migration, and adhesion, was upregulated in PF-EVs (FC = 1.32) but downregulated in plasma (FC = -1.21). It has been identified as part of a high-predictive gene signature (AUC = 0.83) and has been implicated in the promotion of peritoneal adhesion[225]. TAGLN, slightly downregulated in both fluids, stabilizes the myofibroblast phenotype and is present in lesion-associated fibroblast [226, 227]. Modest suppression of TAGLN in EVs may reflect a transient inhibition of fibroblast activity and dynamic remodelling within the lesion microenvironment. Its reduction may reflect transient remodelling activity.

Dysregulation of autophagy has gained increasing attention as a relevant molecular feature of endometriosis, with reduced autophagic activity observed in both eutopic and ectopic endometrium of affected patients[228]. Studies have shown that impaired autophagy may promote the survival and proliferation of endometrial stromal cells while disrupting apoptosis and cellular homeostasis[229, 230]. In this context, particular attention has been drawn to CSNK1A1, which encodes the kinase CK1α-a critical regulator of autophagy via the PTEN/ATG7 pathway[230]. In our study, CSNK1A1 abundance was decreased in EVs derived from both PF (FC = -1.13) and plasma (FC = -1.45) of endometriosis patients. These findings are consistent with previous observations[231] showing that reduced CK1α expression in endometrial tissue correlates with decreased levels of PTEN and ATG7 and impaired autophagy.

In contrast, CBX3, an essential for chromatin compaction and transcriptional repression, showed opposing expression patterns-downregulated in PF-derived EVs (FC = -1.29) and upregulated in plasma-derived EVs (FC = 1.40)-potentially reflecting divergent epigenetic states between the local lesion microenvironment and systemic circulation. CBX3 was significantly downregulated in the endometrium of women with endometriosis in a meta-

analysis of transcriptomic data[180]. Furthermore, genetic association data from the FinnGen project linked a variant near the CBX3 locus with advanced stages of endometriosis (rASRM stage III/IV), supporting its potential role in disease progression[232]. Functionally, reduced levels of CBX3 in PF-derived EVs may reflect a localized failure in maintaining chromatin silencing, possibly promoting aberrant gene expression and endometrial cell plasticity. In contrast, its elevated presence in circulating EVs may signal a compensatory systemic regulatory response.

ABI3BP, associated with senescence, was upregulated in both PF-EVs (FC = 1.36) and plasma-EVs (FC = 1.30). It is overexpressed in decidualizing stromal cells from endometriosis patients and associated with impaired BMP2 signalling and cellular stress[233], with additional disease association evidence within the Harmonizome/DisGeNET database[234]. These findings suggest that ABI3BP may support a dysfunctional, pro-inflammatory endometrial niche that hinders proper decidualization.

In summary, this shared EV proteome reflects key pathological processes-fibrosis, cytoskeletal remodelling, autophagy, and epigenetic dysregulation-across local and systemic compartments. These proteins may aid in developing diagnostic tools, but require validation in larger cohorts, ideally with clinical correlation, staging, and longitudinal follow-up to confirm diagnostic and prognostic value.

5.4. Integrated IFCM-based phenotyping of EVs in endometriosis

In addition to global MS-based proteomic profiling, IFCM was employed as a complementary high-resolution approach enabling single-particle surface phenotyping of EVs in both systemic (plasma) and local (PF) compartments[114, 123] While proteomics provided an untargeted overview of total EV cargo following isolation, IFCM enabled quantitative assessment of predefined surface markers at the level of individual vesicles. The integration of these two analytical strategies allowed not only cross-platform validation of selected findings but also evaluation of how pathway-level proteomic alterations are reflected at the vesicle surface.

The perioperative plasma cohort served as both a biological validation and a methodological proof-of-principle for the IFCM platform. In this initial phase, IFCM analysis was performed exclusively on isolated EV fractions rather than on native plasma. This strategy was deliberately chosen to minimize background interference from abundant soluble plasma proteins and non-vesicular particles and to establish optimal signal-to-noise conditions during assay implementation. By working with purified EV preparations, it was first

confirmed that the selected antibody panel and gating strategy reliably detected vesicle-associated markers in the experimental setting before extending the approach to more complex biofluids.

The coordinated reduction of multiple EV-associated surface markers within 48 hours following laparoscopic removal of endometriotic lesions demonstrated that circulating EV phenotypes are dynamically regulated in response to changes in disease burden as demonstrated by IFCM analysis. Importantly, this effect was not accompanied by a comparable decrease in total EV particle concentration measured by NTA, suggesting that the observed changes primarily reflect shifts in EV surface phenotypes rather than a global reduction in vesicle abundance. Decreased detection of canonical tetraspanins, immune-associated markers, platelet- and erythrocyte-derived proteins, adhesion molecules, and the epithelial marker CD227 (MUC1) suggests that circulating EV populations are closely linked to inflammatory and tissue-remodelling processes associated with active endometriosis. The rapid postoperative changes observed following lesion removal suggest that EV-associated signatures closely track disease activity rather than constituting fixed alterations.

Following this validation phase, IFCM was applied directly to plasma and PF samples without prior EV isolation. From a methodological perspective, the present approach represents an initial optimization of single-EV immunophenotyping using IFCM on the ImageStreamX Mark II platform, applied both to isolated EV preparations and native plasma samples. While the feasibility and analytical sensitivity of this strategy were demonstrated, further studies are required to systematically evaluate reproducibility, detection limits, and robustness across larger and more diverse cohorts. Continued refinement of dilution strategies, fluorescence triggering, and calibration procedures will be essential for the development of a standardized and clinically translatable workflow enabling EV immunophenotyping directly in biological fluids. This shift from controlled validation conditions toward direct analysis in native biofluids reflects a translational perspective, assessing whether disease-associated EV signatures can be detected without extensive preprocessing[124]. From a clinical standpoint, simplified workflows compatible with routine diagnostics are essential for the implementation of EV-based biomarkers. The feasibility of single-particle detection directly in complex biofluids therefore represents a critical conceptual bridge between discovery-driven proteomics and the development of targeted, clinically applicable assays[124].

However, while translationally attractive, the direct transfer of proteomics-identified candidates into IFCM-based panels is not straightforward. In principle, markers emerging

from MS could serve as candidates for targeted surface phenotyping and subsequent clinical screening. In practice, several methodological constraints limit this process. First, the antibody panel used in the present study was not specifically designed for endometriosis but represented a pre-validated multiparametric EV phenotyping panel established in collaboration with an experienced IFCM laboratory. Although several included markers were biologically relevant to inflammatory and stromal pathways implicated in endometriosis, the panel was not optimized to comprehensively capture all disease-specific alterations identified in the proteomic discovery phase.

Second, the development and validation of antibody panels for single-particle EV analysis is inherently challenging. EVs are small structures characterized by low epitope copy numbers, which limits detection sensitivity. Signal intensity depends strongly on fluorophore brightness and labelling efficiency, and complex biofluids contain non-vesicular particles within a similar size range that may interfere with detection. Moreover, antibody preparations may contain vesicular material derived from hybridoma cells or incompletely labelled antibody fractions that act as competitive inhibitors, thereby affecting signal interpretation. For these reasons, extensive qualification of antibody cocktails and consistent use of a standardized panel across experiments are critical to ensure reliability and reproducibility of IFCM measurements.

Third, IFCM detection is restricted to surface-exposed epitopes accessible to antibodies, whereas MS-based proteomics analyses the total protein cargo of isolated EVs, including luminal and membrane-associated proteins. Consequently, many proteins identified as differentially abundant in proteomic analyses may be localized within the vesicle interior and are therefore not amenable to direct surface-based IFCM detection. These methodological differences mean that proteomics quantifies total protein abundance across the EV population, whereas IFCM reflects the proportion of vesicles expressing a given surface marker. Changes in intracellular cargo abundance may therefore not directly translate into altered surface representation, and vice versa. This could be an explanation why only very few proteins were identified both by proteomics and IFCM. the partial overlap observed between proteomic and IFCM findings.

To investigate EV-associated immune signatures in the peritoneal microenvironment, IFCM analysis was performed to compare marker-positive particle concentrations between patients with endometriosis and control subjects. In PF samples, IFCM revealed modulation significantly higher concentrations of antigen presentation-associated molecules, innate immune markers, and stromal and adhesion-related proteins in the endometriosis group

compared with controls, indicating broad immune and microenvironmental activation within the peritoneal niche. Together, these findings indicate that IFCM-based molecular phenotyping of PF samples captures disease-associated modulation of antigen presentation, innate immune activation, and stromal remodelling. The partial overlap with proteomic findings, particularly for HLA-DR, supports the biological relevance of these alterations while highlighting the complementary nature of high-resolution IFCM and MS–based proteomics. Importantly, HLA-DR demonstrated similar changes in both proteomic and IFCM analyses, strengthening the interpretation that enhanced antigen presentation constitutes a central feature of the peritoneal inflammatory microenvironment in endometriosis. The agreement across analytical platforms suggests that this alteration is not confined to a specific EV subpopulation but reflects a robust and biologically relevant shift in vesicle-associated immune signalling.

These findings are consistent with proteomic pathway enrichment analyses indicating dysregulation of IL-17 signalling, ECM-receptor interactions, and ferroptosis-associated processes. The increased surface representation of immune and stromal markers detected by IFCM is mechanistically aligned with inflammatory amplification, immune cell recruitment, and ECM remodelling-processes that are hallmarks of lesion persistence and peritoneal fibrosis. Notably, the broader spectrum of alterations observed in PF compared to plasma is biologically plausible, as PF directly reflects the inflammatory microenvironment surrounding endometriotic lesions, whereas plasma represents a diluted systemic compartment in which only selected EV signatures remain detectable.

In plasma, the observed changes were more selective, yet cross-platform concordance for CD81 and CD227 (MUC1) supports their biological relevance in the systemic compartment. The presence of corresponding alterations across both proteomics and IFCM suggests that these markers may represent more stable and systemically detectable components of disease-associated EV signatures.

Among the systemically detectable markers, CD227 (MUC1) is of particular consideration. In the present cohort, MUC1 demonstrated cross-platform agreement between proteomic profiling and IFCM analysis in plasma, supporting its stability as a systemically measurable EV-associated marker. Given its role as a transmembrane epithelial mucin involved in cell adhesion, barrier integrity, and immune modulation, its presence in circulating EVs may reflect epithelial remodelling processes associated with endometriosis. Notably, in an independent perioperative cohort, circulating MUC1-positive EVs were reduced following surgical removal of endometriotic lesions, providing supportive evidence

that EV-associated MUC1 may dynamically reflect changes in disease burden. Although derived from separate patient groups, the similar direction of these findings strengthens the biological plausibility of MUC1 as a translationally relevant systemic indicator.

Moreover, several proteins were identified in both PF and plasma compartments, supporting the existence of EV-associated molecular features that extend beyond strictly local peritoneal inflammation. Notably, antigen presentation-associated molecules such as HLA-ABC and HLA-DR were detected by MS in both compartments, with HLA-DR additionally confirmed by IFCM in PF. Similarly, the canonical tetraspanin CD81 was identified across both compartments by proteomics and demonstrated cross-platform concordance in plasma, where it also reached differential abundance. Innate and adhesion-related markers including CD14, CD44, and CD90 (THY1) were likewise present in PF and plasma EV proteomes, suggesting shared immune-stromal signalling axes operating at both local and systemic levels. Importantly, the epithelial-associated marker CD227 (MUC1) was detected in both compartments by proteomics and showed similar plasma alterations in IFCM, further supporting its potential relevance as a systemically measurable EV-associated feature. Together, these overlapping signatures indicate that selected EV-associated proteins may reflect systemic adaptations accompanying localized peritoneal inflammation in endometriosis.

The detection of PD-L1 exclusively by IFCM further illustrates the added resolution provided by single-particle phenotyping. A partial concordance was observed between proteomic and IFCM analyses. In plasma, CD227 (MUC1) and CD81 demonstrated consistent alterations across both platforms. In PF samples, HLA-DR was identified as differentially abundant in the proteomic dataset and showed concordant modulation in IFCM analysis. However, several markers that reached statistical significance in IFCM were not classified as DEPs in the proteomic dataset, while conversely, a subset of proteomics-derived DEPs was not detected by IFCM.

These discrepancies are consistent with inherent methodological differences between the two approaches. Proteomic analysis, performed on isolated EV fractions, enables broad and untargeted profiling of total vesicular protein cargo. In contrast, IFCM measurements-conducted on isolated EVs or directly in native biofluids-are restricted to predefined surface markers. Consequently, proteomics reflects total protein abundance, including luminal components, whereas IFCM captures the distribution of surface-exposed epitopes across EV subpopulations. Together, these complementary analytical dimensions provide a more comprehensive understanding of EV biology in endometriosis.

Changes in the proportion of PD-L1-positive EV subpopulations may occur without substantially altering total protein abundance measurable by proteomics. Given the emerging evidence implicating immune checkpoint pathways in the regulation of local immune tolerance and lesion persistence, EV-associated PD-L1 may represent a mechanistically relevant contributor to immune modulation in endometriosis. This observation highlights how single-particle approaches can uncover biologically meaningful subpopulation shifts that remain masked in bulk proteomic analyses.

Overall, the combined use of untargeted proteomics and targeted single-particle IFCM analysis provides a multidimensional characterization of EV alterations in endometriosis. Proteomics captures global pathway-level dysregulation involving immune activation, oxidative stress imbalance, and ECM remodelling, whereas IFCM reveals how selected components of these processes are distributed at the vesicle surface and across EV subpopulations. The broader alterations observed in PF are consistent with its role as the primary inflammatory niche of the disease, while plasma reflects a more selective systemic signature. Together, these findings support a model in which EV-associated molecular signatures are dynamically linked to disease activity and may serve as mechanistically informative and potentially translational biomarkers in endometriosis.

When interpreting these findings, several considerations should be acknowledged. The study cohort predominantly included moderate to severe cases of endometriosis, limiting generalization across the entire clinical spectrum. The moderate sample size and group imbalance, resulting from strict inclusion criteria designed to minimize confounding factors, may have affected statistical power. In the proteomic analysis, only a subset of proteins reached statistical significance after FDR correction. Given the limited protein yield inherent to EV studies and inter-individual variability, stringent correction procedures - while essential to control false discovery - may obscure biologically meaningful signals in smaller cohorts. Additionally, the use of TMT-based labelling, although enabling multiplexed quantitative analysis, is susceptible to ratio compression, potentially underestimating true fold changes and reducing dynamic range. Finally, the present study focused on molecular profiling and did not include functional validation experiments to establish causal relationships between altered EV cargo and disease mechanisms. Moreover, the IFCM analysis was performed using a preselected marker panel comprising canonical EV markers together with selected immune, inflammatory, and vascular-associated proteins. While this panel enabled broad phenotypic characterization of EV-associated particles, it was not specifically designed to capture EV signatures associated with endometriosis and was not informed by the proteomic discovery

results obtained in the present study, which may have limited the ability to detect disease-specific vesicle subpopulations.

Future investigations should expand cohort size and include a broader range of clinical phenotypes, ideally incorporating longitudinal sampling to assess dynamic EV signatures in relation to disease progression and therapeutic response. Development of disease-specific EV surface panels compatible with high-throughput, clinically applicable analysis methods like IFCM, integration with RNA and lipidomic profiling, and functional studies evaluating the impact of EV-associated proteins on recipient cells will be essential to refine mechanistic understanding. Importantly, candidate DEPs identified in the discovery proteomic analysis should be further validated using targeted proteomic approaches and orthogonal methods such as Western blotting or ELISA. Particular attention should be given to proteins localized on the EV surface, as these molecules represent the most accessible targets for downstream phenotyping assays and potential diagnostic applications. Standardization of EV isolation and single-particle detection methodologies, together with integration of EV-derived signatures with imaging and hormonal parameters, will represent critical steps toward the translation of EV-based biomarkers into clinically applicable diagnostic and monitoring tools in endometriosis. In this context, the selection of validated surface-associated EV proteins may enable the development of targeted IFCM panels, providing a translational platform for evaluating EV-based biomarker candidates in larger clinical cohorts and ultimately supporting the development of EV-based diagnostic assays.

Taken together, the findings of the present study demonstrate that EVs represent biologically active carriers of disease-associated molecular information in endometriosis. The combined use of high-resolution proteomics and single-particle IFCM provided complementary insights into both the intracellular cargo and surface phenotypes of EVs in systemic and peritoneal compartments. The observed alterations in immune signalling, ECM remodelling, and oxidative stress-related pathways highlight the multifaceted role of EVs in shaping the inflammatory microenvironment characteristic of the disease.

Importantly, the partial concordance and methodological differences between proteomic and IFCM analyses underscore the complexity of EV biology rather than representing inconsistencies. Instead, they reveal distinct yet interconnected analytical dimensions-quantitative cargo profiling and surface-level subpopulation phenotyping-that collectively enhance our understanding of EV-mediated communication in endometriosis.

By integrating discovery-driven proteomics with targeted single-particle phenotyping, this work establishes a methodological framework for future EV-based biomarker

development and supports the concept that vesicle-associated molecular signatures may serve as mechanistically informative indicators of disease activity. In addition to their potential diagnostic value, the identification of key DEPs and enriched molecular pathways provides new insights into biological mechanisms underlying endometriosis pathogenesis. These findings may also highlight candidate molecules and signalling processes that could be explored as potential targets for future therapeutic interventions aimed at modulating inflammatory responses, ECM remodelling, or immune dysregulation associated with disease progression. Further refinement of disease-specific EV phenotyping panels and validation in larger cohorts will be essential to translate these findings into clinically applicable diagnostic strategies.

5. CONCLUSIONS

Altogether, the results obtained in this study demonstrate that EVs represent biologically informative carriers of molecular signals associated with endometriosis. The combined analytical strategy applied in this work provides a comprehensive framework for the investigation of EV-associated molecular signatures in both systemic and peritoneal compartments. The identification of compartment-specific as well as shared EV-associated proteins linking local peritoneal processes with systemic alterations provides new insight into the molecular landscape of endometriosis. These findings highlight the potential of EV-associated molecules as candidates for further investigation in future studies aimed at improving the molecular characterization of the disease.

The performed studies allow to conclude that:

- EVs can be reliably isolated from both plasma and PF using SEC combined with rigorous characterization approaches, providing high-quality vesicle preparations suitable for downstream proteomic analyses;
- high-resolution quantitative proteomic profiling revealed extensive EV-associated protein landscapes in both biological fluids and, importantly, enabled discrimination between endometriosis patients and controls, with stronger separation observed in PF-derived EVs; notably, one patient with advanced (stage IV) endometriosis exhibited a distinct proteomic profile, suggesting that EV signatures may reflect disease severity, although this observation requires validation in larger cohorts;
- EVs derived from PF capture localized molecular alterations that differ between endometriosis patients and controls, indicating dysregulation of key biological pathways within the peritoneal microenvironment, including immune activation, oxidative stress, iron metabolism, ECM remodelling, and cellular adhesion; these alterations are reflected by the differential abundance of specific EV-associated proteins, including HIC2, which showed the highest FC value despite its currently unclear role, as well as MUC5AC, FAT3, DNMT1 and members of the S100 protein family;
- plasma-derived EVs exhibit differential protein abundance between endometriosis patients and controls, suggesting dysregulation of systemic biological pathways, including intracellular signalling, immune modulation, epithelial remodelling and

vesicle trafficking; these alterations are reflected by specific EV-associated proteins such as PIK3C2A, which showed the strongest upregulation and is involved in vesicle trafficking and intracellular signalling, although its role in endometriosis remains largely unexplored, as well as SDC4, MUC16 and S100A12, supporting their potential relevance in systemic disease responses and their possible utility in minimally invasive assessment of endometriosis;

- a subset of EV-associated proteins was consistently detected in both PF and plasma, indicating the presence of shared molecular mechanisms linking local lesion biology with systemic disease manifestations;
- the integration of quantitative proteomics with IFCM applied in an exploratory pilot cohort, enabled complementary characterization of EV cargo composition and vesicle surface phenotypes, providing a multidimensional perspective on EV-associated alterations in endometriosis;
- several EV-associated proteins and surface markers demonstrated concordant alterations across analytical platforms, including MS–based proteomics and IFCM, with consistent changes observed for CD81 and MUC1 (CD227) in plasma-derived EVs and HLA-DR in PF-derived EVs, supporting their biological relevance and highlighting their potential value for further biomarker-oriented investigations; in addition, several markers, such as CD14, CD44, CD31, CD90, CD82, CD9 and HLA-ABC, were detected across analytical platforms, although not always consistently across compartments, and did not meet differential abundance criteria, indicating partial overlap between analytical platforms.

Altogether, the results obtained in this study demonstrate that EVs represent biologically informative carriers of molecular signals associated with endometriosis. The combined analytical strategy applied in this work enables comprehensive characterization of EV-associated molecular signatures across both local and systemic compartments, providing insight into the mechanisms linking peritoneal processes with systemic alterations and contributing to a deeper understanding of the molecular landscape of the disease.

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8. ATTACHMENTS

8.1. Study approval of the Local Bioethics Committee

Komisja Bioetyczna
przy Warszawskim Uniwersytecie Medycznym

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Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym
w dniu 03 lipca 2023 r. po zapoznaniu się z wnioskiem

Dr hab. n.med.Jacek Sieńko,
Klinika Położnictwa i Ginekologii,
ul. Karowa 2, 00-315 Warszawa

dotyczącym: wyrażenia opinii w sprawie badania pt. "Poszukiwanie potencjalnych markerów w procesie diagnostycznym ,ocenie progresji oraz skuteczności leczenia endometriozy,w tym udział pęcherzyków zewnątrzkomórkowych oraz egzosomalnej arginazy w dysfunkcji układu immunologicznego w endometriozie."

- Badanie może być prowadzone wyłącznie w okresie obowiązywania polisy ubezpieczeniowej.

wyraża następującą
o p i n i ę

- stwierdza, że jest ono dopuszczalne i zgodne z zasadami naukowo-etycznymi*.
- ~~stwierdza, że jest ono niedopuszczalne i niezgodne z zasadami naukowo-etycznymi.*~~

Uwagi Komisji – *verte*

Komisja działa na podstawie regulaminu działania Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym, przyjętego zarządzeniem Rektora WUM nr 200/2022 z dnia 18 października 2022 r. oraz przepisów prawa powszechnie obowiązującego, w tym przede wszystkim ustawy z dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty, ustawy z dnia 6 września 2001 r. Prawo farmaceutyczne, ustawy z 22 kwietnia 2022 r. o wyrobach medycznych oraz prawa międzynarodowego lub unijnego, a także odpowiednich norm naukowych lub zawodowych.

Przewodnicząca Komisji Bioetycznej

Prof. dr hab. n. med. Magdalena Kuźma-Kozakiewicz

*niepotrzebne skreślić