Extracellular vesicles released by uterine fibroid cells: modulation by omega-3 fatty acids and impact on recipient myometrial cells

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Objective: To study the effects of extracellular vesicles (EVs) derived from primary myometrial and leiomyoma cells, as well as suitable cell lines, and to evaluate the release of EVs after omega-3 fatty acid treatment and their impact on fibronectin protein expression in recipient myometrial cells.
Design: Case-control laboratory study.
Setting: University institute and university hospital.
Subjects: Patients with uterine fibroids.
Exposure: Primary cells were obtained from uterine tissue samples (leiomyoma and healthy myometrium) of premenopausal women (41–49 years) undergoing surgery. Immortalized myometrial (A00-9) and leiomyoma (A00-10) cell lines were also used.
Main outcome measure: Cross-sectional in vitro study comparing untreated and omega-3-treated primary and immortalized myometrial and leiomyoma cells. Extracellular vesicles were isolated after 48 hours of treatment, characterized by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), and then used to treat myometrial cell lines. Fibronectin expression was measured in recipient cells.
Results: Extracellular vesicles were isolated and fractionated into small (s) and large (l) EVs. Small extracellular vesicle release was significantly induced by eicosapentaenoic acid treatment. These vesicles derived from leiomyoma cells showed a trend toward increased fibronectin expression in recipient myometrial cells, whereas EVs from omega-3-treated leiomyoma cells showed attenuated effects. Those from myometrial cells, treated or untreated, did not alter fibronectin expression. Results were consistent across biological replicates (n = 3).
Conclusion (s): These data suggest that leiomyoma-derived EVs contribute to fibrotic changes in surrounding myometrium and that omega-3 fatty acids can modulate EV release and EV-mediated profibrotic signaling. This may represent a potential proof-of-concept strategy for managing fibroid-associated fibrosis, although further in vivo validation is required. (F S Sci® 2026;■:■–■. ©2026 by American Society for Reproductive Medicine.)

Key Words: Extracellular vesicles (EVs), uterine fibroids, myometrium, fibronectin, omega-3 fatty acids

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F&S SCIENCE CLINICAL QUICK TAKE

What clinical problem is addressed by these studies?

- Uterine fibroids are associated with excessive extracellular matrix deposition and fibrosis, contributing to symptoms and impaired fertility.
- The mechanisms driving fibrotic changes in surrounding myometrium remain poorly understood.

What are the key findings?

- Leiomyoma-derived extracellular vesicles increase fibronectin expression in recipient myometrial cells, suggesting a profibrotic paracrine effect.
- Omega-3 fatty acids enhance EV release but attenuate their profibrotic activity.

How do these findings apply to human fertility or the reproductive process?

- Modulation of EV-mediated signaling by omega-3 fatty acids may reduce fibrotic remodeling of the uterus.
- This may potentially improve uterine function and fertility outcomes.

All cell types can produce nanosized particles known as extracellular vesicles (EVs), which mediate intercellular communication (1, 2). These are enclosed by a lipid bilayer and transport a wide range of biologically active molecules, including proteins, lipids, and nucleic acids (3, 4). These vesicles can be broadly classified into 3 subtypes on the basis of their cellular origin: exosomes, microvesicles, and apoptotic bodies. Additionally, EVs are categorized as large (l) or small (s) based on their size (5). The functional roles of EVs have been widely documented in various physiological and pathological conditions, including cancer, inflammatory diseases, and tissue remodeling. Extracellular vesicles are increasingly recognized as key regulators of cellular behavior, facilitating the transfer of genetic information, proteins, and signaling molecules between cells (6, 7). These mechanisms emphasize their importance in both maintaining normal tissue homeostasis and driving disease processes.

Leiomyomas, also known as uterine fibroids, are benign smooth muscle tumors of the uterus and a major cause of gynecologic morbidity, often associated with heavy menstruation, pelvic pain, and infertility. Despite their high prevalence, the pathogenesis of leiomyomas remains poorly understood. Although factors such as African descent and genetic mutations (e.g., MED12, HMGA2) are established risk factors for multiple or solitary fibroids, additional mechanisms, such as EV-mediated paracrine signaling, may also contribute to the multifocal development and growth of these tumors (8).

Emerging evidence has highlighted the role of EVs in the biology of leiomyomas. Indeed, exosomes isolated from a human uterine leiomyoma cell line have been characterized, revealing their protein, ribonucleic acid (RNA), and lipid cargo. These exosomes have been shown to influence the behavior of endometrial vascular endothelial cells, promoting angiogenesis and enhancing the tumor microenvironment (9). Furthermore, EVs are thought to play a role in extracellular matrix (ECM) remodeling by transporting matrix-modulatory enzymes such as matrix metalloproteinases (MMPs), which are critical for leiomyoma growth and stiffness (10, 11). Therefore, EV-mediated paracrine mechanisms could also induce changes in neighboring myometrial cells, promoting fibroid initiation or progression. Although the impact of EVs on primary myometrial cells has not yet been studied, preliminary data from other tumor systems suggest that EVs can profoundly alter the cellular phenotype of recipient cells, including their proliferation, migration, and ECM deposition (12, 13). In leiomyomas, such changes may contribute to the dense and fibrotic nature of the tumors (14). Within a dense and stiff ECM environment typical of fibroids, long-distance diffusion of EVs is likely restricted. We hypothesize that EVs predominantly act in a paracrine fashion on immediate neighboring cells such as mature smooth muscle cells and resident stem/progenitor cells, or diffuse through localized microchannels.

In our recent studies, we explored the impact of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on fibrotic processes in uterine leiomyoma cells. Treatment with EPA and DHA induced a reduction in monounsaturated fatty acids (MUFA) and an increase in polyunsaturated fatty acids (PUFA) in both myometrial and leiomyoma cells. Although these treatments maintained the liquid-crystalline phase of the cell membranes, they resulted in decreased membrane fluidity, suggesting structural remodeling. These membrane alterations influenced key cellular signaling pathways, particularly those related to lipid metabolism and mechanical signaling, as reflected by the downregulation of genes such as ABCG1, ABCA1, AKAP13, FAK, and CYP11A1 (15). Furthermore, omega-3 fatty acids reduced collagen deposition in uterine leiomyomas, an effect that could mitigate ECM remodeling and fibrotic progression, key contributors to ECM stiffness. Furthermore, modulation due to omega-3 treatment at the lipid levels and so up to the cell membrane in myometrial and leiomyoma cells also been reported (16).

The influence of EVs on immune cells within the uterine microenvironment has also garnered significant attention. Extracellular vesicles released by leiomyoma cells could modulate immune responses by suppressing antitumor immunity or promoting a proinflammatory state, both of which can support tumor persistence and growth (17, 18). Despite these advances, significant gaps remain in understanding the exact role of EVs in leiomyoma development. These findings prompted our research group to investigate this phenomenon further.

Our research aimed to characterize EVs produced by both primary myometrial and leiomyoma cells, and suitable cell

lines, using nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and western blotting. We also evaluated the release of EVs after omega-3 fatty acids treatment and used EVs from cells treated with and without omega-3 fatty acids to assess their effects on recipient myometrial cell lines. Specifically, we evaluated the impact of EVs derived from both untreated and omega-3 pretreated myometrial and leiomyoma cell lines on fibronectin protein expression in recipient myometrial cells.

MATERIALS AND METHODS

Drugs and chemicals

Both EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) and DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid) were purchased from Sigma-Aldrich (St. Louis, MO). These compounds were initially dissolved in absolute ethanol at a concentration of 30 mM, followed by dilution in the appropriate culture medium to achieve a final concentration of 50 μM at the time of cell treatment. The final ethanol concentration in the culture medium was kept below 0.1% to minimize any potential effects of ethanol on cell viability and function.

Clinical variables

The following clinical variables were collected: age (years), associated symptoms (heavy menstrual bleeding, compressive symptoms, or pelvic pain), International Federation of Gynecology and Obstetric (FIGO) classification (19), type of surgery (myomectomy or hysterectomy), and histopathology results.

Leiomyoma and myometrial tissue collection and primary cell cultures

The study was conducted on premenopausal Caucasian women aged 41–49 years (n = 3), all of whom had symptomatic uterine fibroids. None of the participants had received hormonal therapy in the 3 months preceding surgery, thereby minimizing potential confounding effects of hormonal treatment on tissue characteristics. The research protocol was approved by the Human Investigation Committee of Marche (protocol number 2015 0486), and written informed consent was obtained from all patients before tissue collection. Tissue samples were collected during hysterectomy or laparotomic myomectomy procedures.

The procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Myometrial and fibroid tissue samples were promptly collected in Hanks' balanced salt solution (HBSS; Sigma-Aldrich) immediately after surgery to preserve cellular integrity. The tissues were thoroughly washed twice with Dulbecco's phosphate-buffered saline (PBS) (Corning, New York, NY) to remove any residual blood and contaminants.

The tissue pieces were then enzymatically digested with 0.1% collagenase type 8 (Serva Electrophoresis GmbH, Heidelberg, Germany) in serum-free Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich), supplemented with 1% penicillin–streptomycin (Corning), 50 μg/L gentamicin (Corning), and 1% amphotericin B (Corning). The digestion process took place at 37 °C for 3–5 hours in a water bath with periodic manual shaking to ensure uniform tissue breakdown. After digestion, the resulting cell suspension was centrifuged at 1,200 rpm for 10 minutes, and the supernatant was discarded. The cell pellet was then resuspended and washed once with fetal bovine serum (FBS; Corning) to neutralize the collagenase. The cells were plated in DMEM containing 10% FBS, 1% penicillin–streptomycin (Corning), 50 μg/L gentamicin (Lonza), and 1% amphotericin B (Corning). The culture was maintained in a humidified incubator at 37 °C with a 95% air 5% CO_2 atmosphere.

The culture medium was changed after 48–72 hours to remove nonadherent cells. After this initial period, the medium was refreshed twice weekly. Cell purity was routinely assessed through immunocytochemical staining for α-smooth muscle actin (α-SMA), a specific marker of smooth muscle cells, using a monoclonal mouse anti-α-SMA antibody (Sigma-Aldrich).

To avoid phenotypic changes and maintain cellular homogeneity, cells were used only up to passage 5. The lower passage numbers helped preserve the characteristics of the original tissue and ensured the maintenance of the myometrial or fibroid smooth muscle phenotype during experimental procedures. All experiments were performed using these early-passage cells to ensure reproducibility and minimize variability in cellular behavior due to extended culturing.

Cell lines culture

The myometrial (A00-9) and leiomyoma (A00-10) cell lines were provided by William H. Catherino, M.D., Ph.D. (Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland). The immortalization of the primary myometrial and leiomyoma cells was obtained with the transfection of human papillomavirus type 16 as described by Malik et al. (20). Cells were cultured in DMEM-F12 (Corning) supplemented with 10% FBS (Corning), 1% antibiotic (penicillin–streptomycin; Corning), 1% fungizone (amphotericin B; Corning), and 1% glutamine (Corning) at 37 °C in 95% air 5% CO_2.

Treatment with omega-3

Myometrial and leiomyoma primary cells and cell lines were detached from T75 flasks (Corning) using trypsin (Corning) and then counted. Cells were treated with omega-3 fatty acids for 48 hours in medium supplemented with exosome-depleted FBS. After treatment, microvesicles and exosomes were isolated from the conditioned medium of treated cells.

Extracellular vesicle isolation

Conditioned media from untreated and omega-3-treated myometrial and leiomyoma primary cells and cell lines were collected after 48 hours of treatment in EV-depleted media. Conditioned media were only used for EV collection. The EV isolation was performed following the guidelines

reported in Turchinovich et al. (21). Briefly, the medium was first cleared by centrifugation for 15 minutes at 1,000 × *g* to eliminate cell contamination. Supernatants were further centrifuged for 20 minutes at 12,000 × *g* and subsequently for 20 minutes at 18,000–20,000 × *g*. The resulting supernatants were filtered through a 0.22 μm filter, and then the EVs were pelleted by ultracentrifugation at 110,000 × *g* for 70 minutes. The EV pellets were washed in PBS, pelleted again, and resuspended in PBS.

Nanoparticle tracking analysis

The EV preparations were characterized according to the guidelines of the International Society for Extracellular Vesicles (5, 22). The EV size and concentration were measured by NTA. Isolated EVs were diluted to 1:100 in PBS and stored at −80 °C for further analysis. Nanoparticle tracking analysis measurements were performed with a NanoSight LM12 (NanoSight, Malvern Instruments Ltd., Malvern, United Kingdom), equipped with a sample chamber with a 405-nm laser. The samples were injected into the sample chamber with sterile syringes until the liquid reached the tip of the nozzle. All measurements were performed at a controlled temperature of 22 °C (± 0.5 °C). Camera level, threshold, and sensor frame per second parameters were kept consistent across all acquisitions.

Transmission electron microscopy observation

The morphological characterization of vesicles was performed using TEM, following a standard negative staining protocol. Extracellular vesicles were immediately fixed in 2.5% glutaraldehyde for 30 minutes, then deposited onto 200-mesh Formvar carbon-coated grids (Agar Scientific Ltd., Stansted, United Kingdom) for 10 minutes to allow adsorption. Subsequently, the grids were stained with a 2% sodium phosphotungstate solution for 1 minute. Excess stain was carefully removed using filter paper to prevent artifacts. The samples were then visualized using a Philips CM10 transmission electron microscope, operated at 80 kV, to assess vesicle morphology and structural integrity.

Myometrial cell lines treatment with EVs

Extracellular vesicles were isolated from myometrial and leiomyoma cell lines, both untreated and pretreated with omega-3 fatty acids, to assess their effects on fibronectin protein expression in recipient myometrial cell lines.

In detail, cells were detached from T75 flasks (Corning) using trypsin (Corning). After detachment, cells were either left untreated or treated with EPA and DHA. Both exosomes and microvesicles were subsequently isolated from the conditioned medium. The isolated EVs, normalized by particle number on the basis of NTA counts to ensure a standardized dosage, were then used to treat myometrial cell lines cultured in 6-well plates (Corning). Recipient cells were incubated with the vesicles for 48 hours in medium supplemented with exosome-depleted FBS. After the treatment period, cells were lysed using TRIzol reagent (Sigma-Aldrich, St. Louis, MO) to extract proteins. The lysates were stored at −80 °C.

Protein extraction and western blotting

Proteins were extracted from recipient myometrial cells using TRIzol reagent (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. Protein concentrations were determined by the Bradford assay (Sigma-Aldrich). Equal amounts of protein were loaded onto 4%–12% NuPAGE gels (Invitrogen, Life Technologies, Carlsbad, CA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were then transferred onto 0.2 μm nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA), and successful transfer was confirmed by Ponceau S staining (Sigma-Aldrich). Membranes were blocked with EveryBlot blocking buffer (Bio-Rad) for 7 minutes at room temperature and incubated overnight at 4 °C with primary antibodies: mouse monoclonal anti-fibronectin (1:100; Sigma-Aldrich), mouse monoclonal anti-β-actin (1:1,000; Sigma-Aldrich), rabbit monoclonal anti-CD9 (1:1,000 clone D801A; Cell Signalling, Danvers, MA), rabbit polyclonal anti-TSG101 (1:1,000; Merck, Milan, Italy), and rabbit polyclonal anti-Calnexin (1:1,000; Merck). After 4 washes with 1X TBST (tris-buffered saline with 0.1% Tween 20), membranes were incubated for 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:1,500; Sigma-Aldrich). Immunoreactive bands were detected using clarity western ECL substrate (Bio-Rad) and imaged on a ChemiDoc system (Bio-Rad). Band intensities were quantified using ImageJ (version 1.49n; NIH) and normalized to β-actin to assess relative protein expression levels.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.5.0 for macOS (GraphPad Software, San Diego, CA). Owing to the limited number of observations available in each experimental group, no inferential statistical analyses were conducted for the western blotting data. These data are therefore presented descriptively and should be interpreted as exploratory, without formal hypothesis testing or estimation of variability.

RESULTS

The present study aims to

- isolate EVs derived from myometrial and leiomyoma cells fractioned into large EVs (lEVs) and small EVs (sEVs) and characterized to validate the purity;
- evaluate the EVs secretion after omega-3 fatty acids (DHA and EPA) treatments; and
- elucidate the role of leiomyoma EVs, specifically focusing on their capacity to modulate fibronectin expression in recipient myometrial cells and on how this interaction is influenced by omega-3 fatty acids (DHA and EPA).

To enhance the translational relevance of our findings, EVs were isolated from both immortalized cell lines and primary cultures. Cells were subjected to treatment with vehicle (ethanol), DHA, or EPA, and conditioned media were collected after a 24–48 hours incubation period.

EV isolation and characterization

Extracellular vesicles were fractionated into large EVs (lEVs) and small EVs (sEVs) using a differential serial ultracentrifugation protocol, with pellets recovered at 15,000 × *g* and 110,000 × *g*, respectively. Subsequent characterization validated the purity and identity of the isolates.

Western blot analysis revealed a marked enrichment of the canonical sEV markers TSG101 and CD9, with no detectable signal for the endoplasmic reticulum protein Calnexin, confirming the high purity of the sEV preparation. In contrast, lEVs showed lower levels of TSG101 and CD9 and were positive for Calnexin, demonstrating both the effectiveness of the ultracentrifugation-based separation and the presence of endoplasmic reticulum-derived components within the lEV fraction. Notably, marker expression profiles were consistent across both myometrial- and leiomyoma-derived EVs.

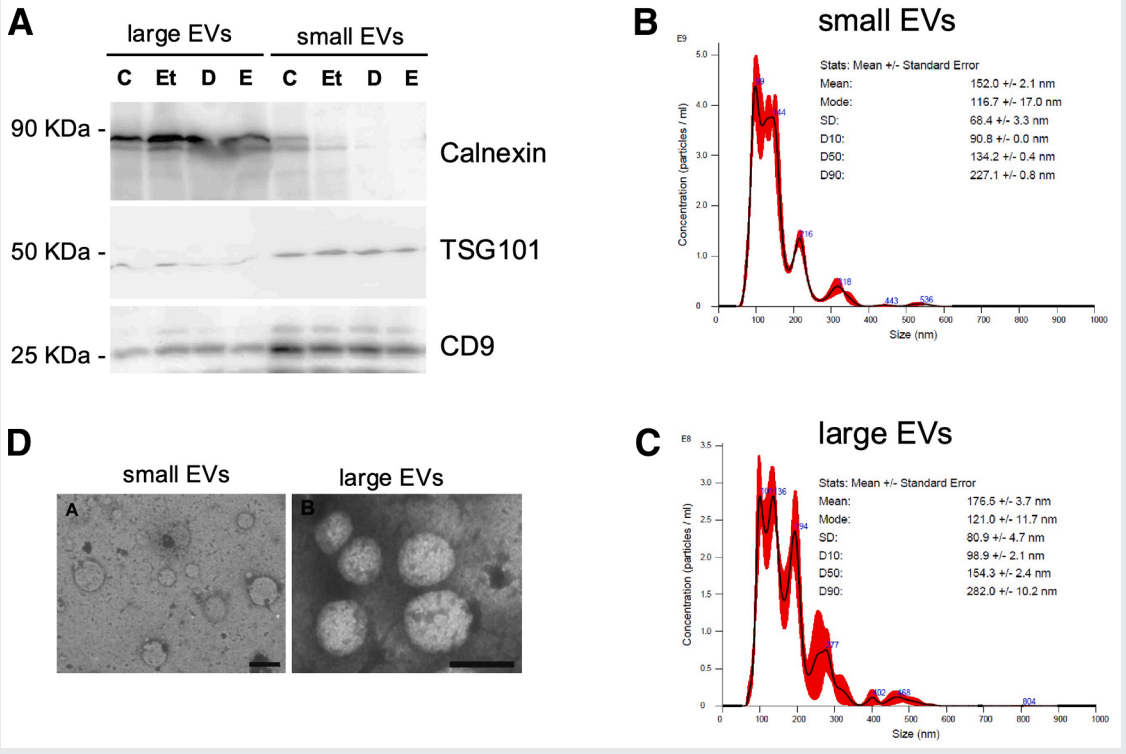
Regarding physical properties, NTA determined a mean hydrodynamic diameter of approximately 150 nm for sEVs, whereas lEVs exhibited a larger and more heterogeneous size distribution (mean ~170 nm). Although these experimental size ranges slightly overlap and appear narrower than some canonical EV definitions, they reflect the specific differential ultracentrifugation protocol and cellular sources used in this study.

Transmission electron microscopy corroborated the vesicular nature of the samples, showing typical membrane-bound structures with average diameters of ~90 nm for sEVs and ~150 nm for lEVs. Collectively, these data demonstrate the isolation of well-characterized, reproducible vesicle populations suitable for downstream functional assays (data not shown, results included in Figures 1 and 2 referred in the following paragraph).

Clinical characteristics

The study was conducted on premenopausal Caucasian women aged 41–49 years (n = 3). The 3 included patients underwent surgery due to symptomatic uterine fibroids. All patients presented with heavy menstrual bleeding, and 1 had compressive symptoms. Two patients aged 41 and 43 years underwent laparotomic myomectomy for a single fibroid (9 cm, FIGO 5 and 10 cm, FIGO 6), and 1 aged 49 years underwent laparotomic hysterectomy for multiple fibroids (10 cm, FIGO 6; 8 cm FIGO 5; and 5 cm FIGO 5). Histopathologic

FIGURE 1



Isolation and characterization of extracellular vesicles (EVs) from myometrial cells. (**A**) EVs were isolated and characterized by western blot, confirming specific enrichment in TSG101, CD9, and Calnexin for lEVs and sEVs. (**B, C**) NTA showed size distribution and concentration of small (sEVs) and large EVs (lEVs), while (**D**) TEM revealed their typical rounded, membrane-bound morphology (sEVs bar = 100 nm; lEVs bar = 200 nm). *Curves* represent averaged data from replicates. EVs = extracellular vesicles; lEVs = large extracellular vesicles; NTA = nanoparticle tracking analysis; sEVs = small extracellular vesicles; TEM = transmission electron microscopy.

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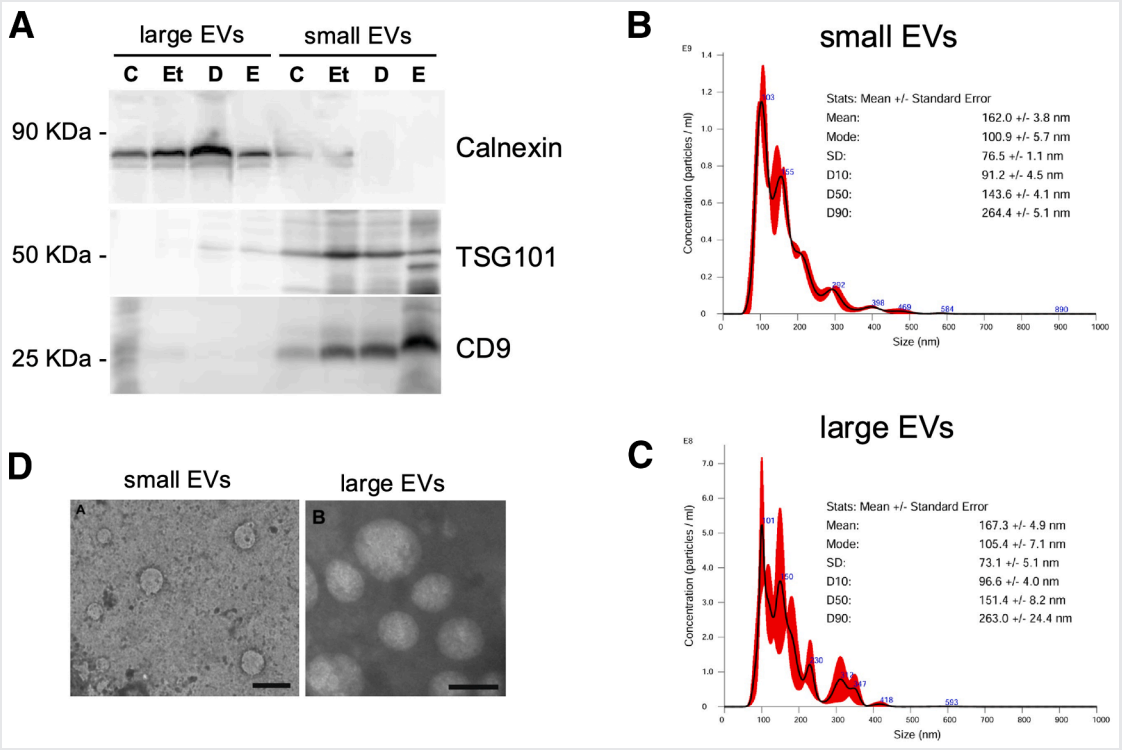
FIGURE 2



Isolation and characterization of extracellular vesicles (EVs) from leiomyoma cells. (**A**) Extracellular vesicles were isolated and analyzed by western blot, confirming the specific enrichment of TSG101, CD9, and Calnexin in both lEVs and sEVs. (**B, C**) Nanoparticle tracking analysis showed size distribution and concentration of small (sEVs) and large EVs (lEVs), whereas (**D**) TEM revealed their typical rounded, membrane-bound morphology (sEVs bar = 100 nm; lEVs bar = 200 nm). *Curves* represent averaged data from replicates. EVs = extracellular vesicles; lEVs = large extracellular vesicles; sEVs = small extracellular vesicles; TEM = transmission electron microscopy.

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evaluation showed the typical features of uterine fibroids. No evidence of hyaline degeneration was found, nor histological variants (cellular, symplastic, mytotically active, hydropic, lipoleiomyoma, epithelioid, myxoid, or dissecting leiomyoma).

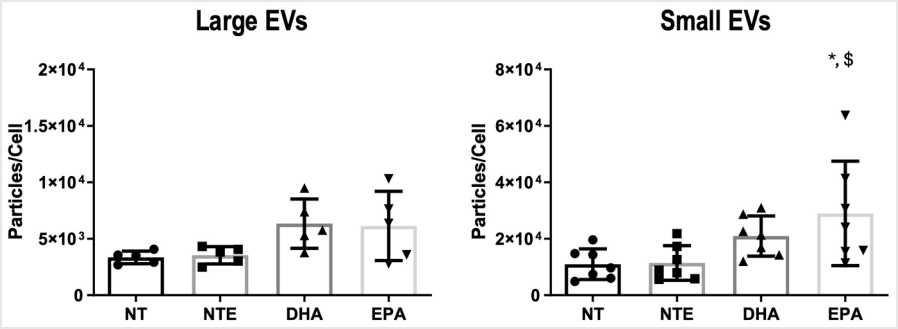
EV isolation and characterization after omega-3 fatty acid treatment in myometrial and leiomyoma cells

Extracellular vesicles were isolated from myometrial and leiomyoma cell cultures after treatment with omega-3 fatty acids or untreated. We used primary cells derived from 3 different patients and myometrial and leiomyoma cell lines. Conditioned media were collected after 24–48 hours of incubation and subjected to a serial ultracentrifugation protocol, enabling the separation of 2 distinct EV subpopulations: large EVs (lEVs), pelleted at 15,000 × *g*, and small EVs (sEVs), pelleted at 110,000 × *g*.

Extracellular vesicles were isolated from conditioned media derived from control (C), ethanol vehicle (Et), DHA (D), and EPA (E) treatments. Western blot analysis confirmed the presence of canonical sEV markers Tsg101 and CD9, and the absence of the endoplasmic reticulum marker Calnexin, in sEVs released by both myometrial (Fig. 1A) and leiomyoma (Fig. 2A) cells under all experimental conditions. In contrast, lEVs exhibited lower expression levels of Tsg101 and CD9, supporting the efficacy of the ultracentrifugation protocol in discriminating between EV subtypes.

No significant differences were observed in EV marker expression between myometrial- and leiomyoma-derived vesicles. Nanoparticle tracking analysis revealed a modest difference in hydrodynamic diameter between sEVs and lEVs: sEVs displayed a mean diameter of approximately 150 nm, whereas lEVs exhibited greater size variability with an average diameter of 170 nm (Fig. 1B and C, myometrial; Fig. 2B and C, leiomyoma). Transmission electron microscopy with negative staining confirmed the vesicular morphology of the isolated EVs, which appeared as membrane-bound, rounded structures. More specifically, sEVs measured approximately 90 nm in diameter, whereas lEVs were around 150 nm (Fig. 1D, myometrial; Fig. 2D, leiomyoma). Furthermore, omega-3 fatty acid treatments were found to induce a trend in increased EV secretion, as detailed in Figure 3 for leiomyoma cells. Both DHA and EPA were found to stimulate lEV accumulation, with this effect being

FIGURE 3



Quantification of extracellular vesicle release after omega-3 fatty acid treatments. Extracellular vesicle secretion (lEVs and sEVs) was quantified using NTA in control cells (NT), ethanol vehicle (NTE), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) treated cells. Similar results were found in both in cells isolated from different patients and in cell lines. One-way ANOVA test followed by Tukey's multiple comparisons test $P<.05$ NT vs. EPA (*), NTE vs. EPA ($). ANOVA = analysis of variance; lEVs = large extracellular vesicles; NTA = nanoparticle tracking analysis; sEVs = small extracellular vesicles.

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even more abundant for sEVs. We have reported similar results both in cells isolated from different patients and in cell lines. Therefore, we conducted the experiments reported below using cell lines. Extracellular vesicles from myometrial cells reported similar results (data not shown).

Modulation of fibronectin expression in recipient myometrial cell lines after treatment with EVs:

The modulation of fibronectin expression in recipient myometrial cell lines was assessed after treatment with the following EVs:

1. Small EVs derived from myometrial cells untreated or pre-exposed to omega-3 fatty acids
2. Large EVs derived from myometrial cells untreated or pre-exposed to omega-3 fatty acids
3. Small EVs derived from leiomyoma cells untreated or pre-exposed to omega-3 fatty acids
4. Large EVs derived from leiomyoma cells untreated or pre-exposed to omega-3 fatty acids

The results are summarized as follows:

1. Fibronectin expression modulation after treatment with small EVs derived from myometrial cells

Small EVs isolated from myometrial cells treated or untreated with omega-3 fatty acids did not induce any changes in fibronectin expression in recipient myometrial cell lines (Fig. 4).

2. Fibronectin expression modulation after treatment with large EVs derived from myometrial cells

Large EVs isolated from myometrial cells either untreated and pretreated with omega-3 fatty acids did not induce an evident alteration in the expression of fibronectin in recipient myometrial cell lines, as assessed by western blot analysis (Fig. 5).

3. Fibronectin expression modulation after treatment with small EVs derived from leiomyoma cells

Small EVs derived from leiomyoma cell lines were associated with a trend toward increased fibronectin expression in recipient myometrial cells, as shown in Figure 6. Conversely, if leiomyoma cells have been treated with omega-3 fatty acids, their small EVs had attenuated ability to induce fibronectin expression in recipient myometrial cells.

4. Fibronectin expression modulation after treatment with large EVs from leiomyoma cells

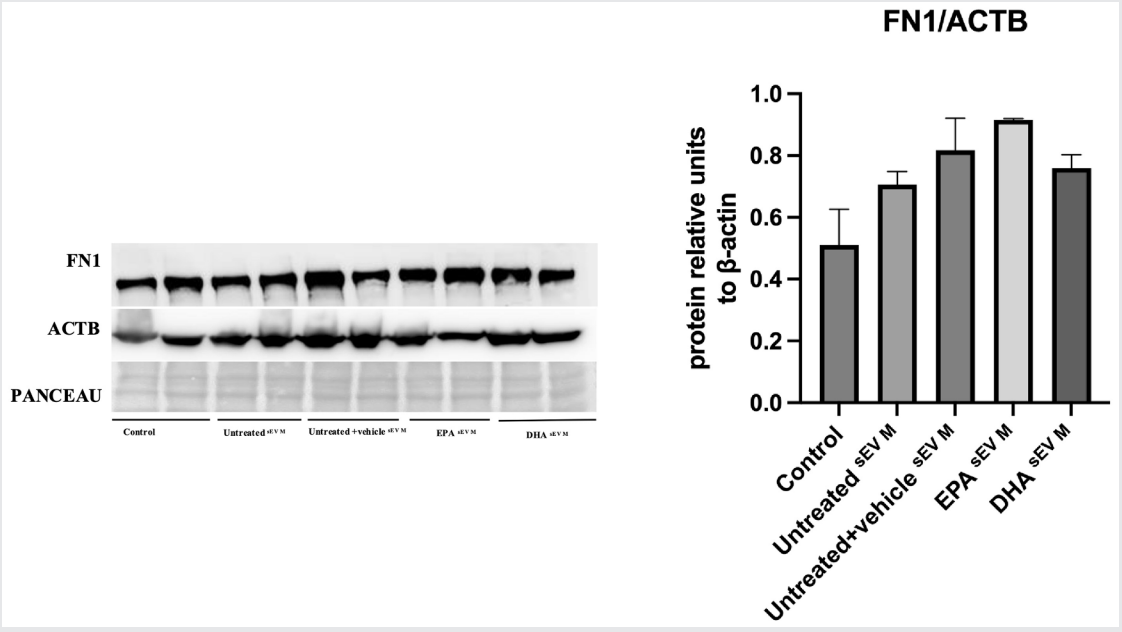
Large EVs derived from leiomyoma cell lines showed a trend toward increased fibronectin expression in recipient myometrial cells. Fibronectin expression was absent or showed a clear trend toward attenuation after treatment with large EVs derived from leiomyoma cells pretreated with omega-3, compared with untreated controls (Fig. 7).

DISCUSSION

In the present study, we isolated EVs from primary myometrial and leiomyoma cells, as well as from cell lines, with and without pretreatment with omega-3 fatty acids (EPA and DHA). Nanoparticle tracking analysis confirmed the size distribution and concentration of these EVs. Both small EVs (30–150 nm) and large EVs (100–1,000 nm) were isolated, with omega-3 fatty acid treatment influencing EV concentration and cargo composition without altering the overall size distribution.

We observed that EVs derived from leiomyoma cells significantly upregulated fibronectin expression in recipient myometrial cells. This suggests that EVs from fibroid cells contribute to the uterine fibrotic environment, aligning with previous studies showing that fibroid EVs mediate endometrial function (9).

FIGURE 4



Western blot analysis of fibronectin expression in recipient myometrial cells exposed to small extracellular vesicles (sEVs). Recipient myometrial cells were either left untreated (control) or treated with sEVs isolated from myometrial cell lines under different conditions: sEVs from untreated myometrial cells (Control sEV M), sEVs from eicosapentaenoic acid-treated myometrial cells (EPA sEV M), and sEVs from docosahexaenoic acid-treated myometrial cells (DHA sEV M). Fibronectin protein expression was assessed by western blotting and normalized to the appropriate loading control. DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; sEVs = small extracellular vesicles.

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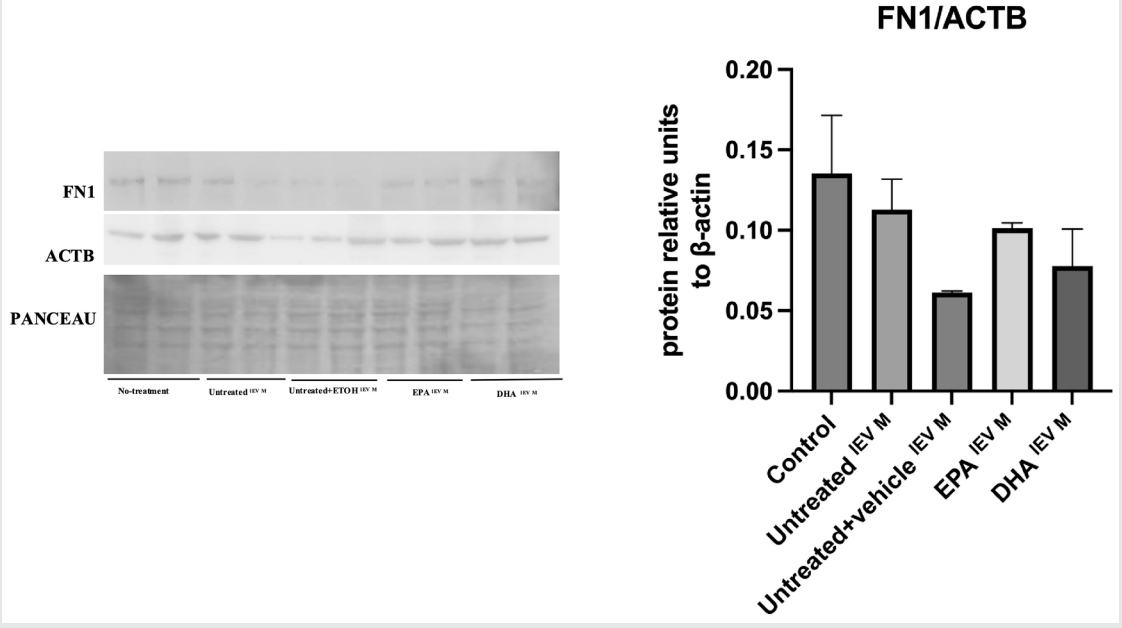
FIGURE 5



Western blot analysis of fibronectin expression in recipient myometrial cells exposed to large extracellular vesicles (lEVs). Recipient myometrial cells were either left untreated (Control) or treated with lEVs isolated from myometrial cell lines under different conditions: lEVs from untreated myometrial cells (Control lEV M), lEVs from eicosapentaenoic acid-treated myometrial cells (EPA lEV M), and lEVs from docosahexaenoic acid-treated myometrial cells (DHA lEV M). Fibronectin protein expression was assessed by western blotting and normalized to the appropriate loading control. DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; lEVs = large extracellular vesicles.

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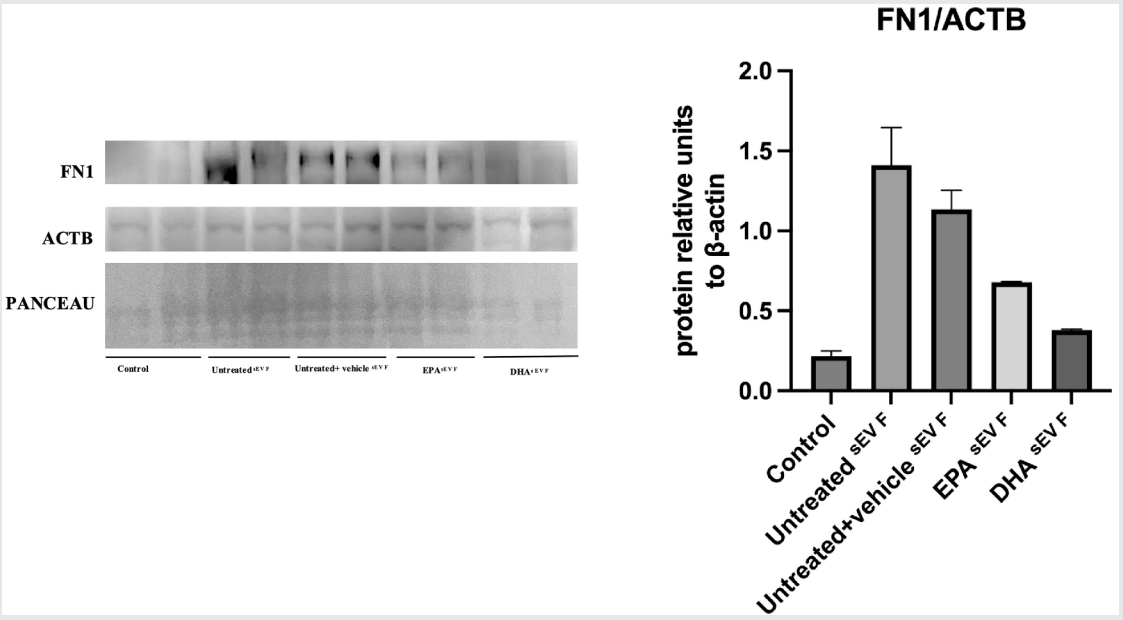
FIGURE 6



Western blot analysis of fibronectin expression in recipient myometrial cells exposed to small extracellular vesicles (sEVs) derived from leiomyoma cell lines. Recipient myometrial cells were either left untreated (Control) or treated with sEVs isolated from leiomyoma cell lines under different conditions: sEVs from untreated leiomyoma cells (Control sEV F), sEVs from eicosapentaenoic acid-treated leiomyoma cells (EPA sEV F), and sEVs from docosahexaenoic acid-treated leiomyoma cells (DHA sEV F). Fibronectin protein expression was assessed by western blotting and normalized to the appropriate loading control. DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; sEVs = small extracellular vesicles.

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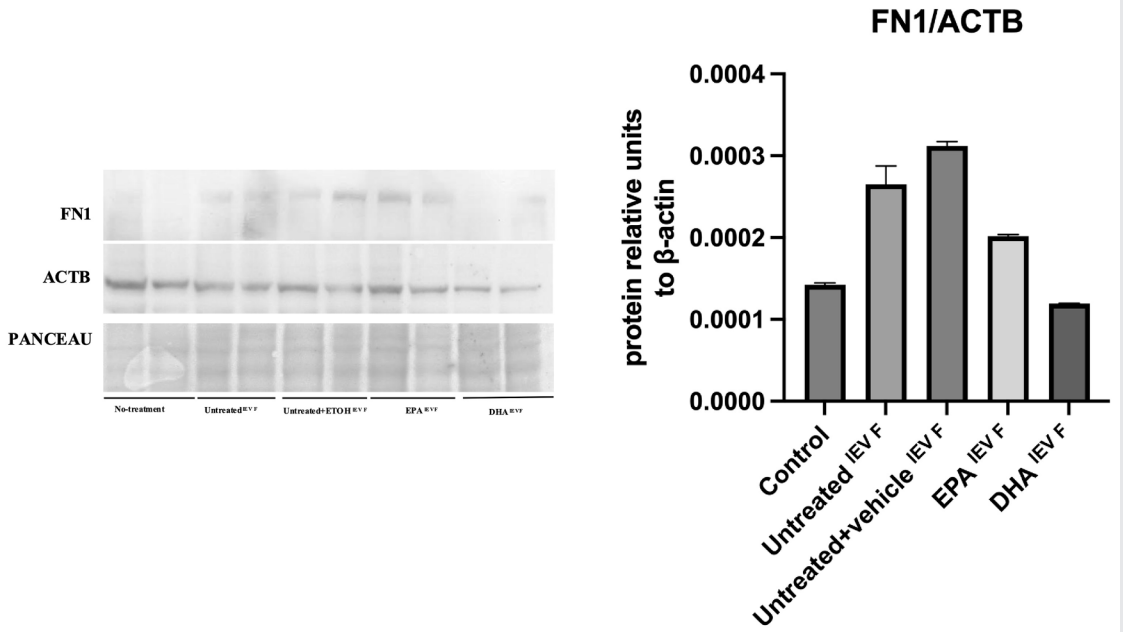
FIGURE 7



Western blot analysis of fibronectin expression in recipient myometrial cells exposed to large extracellular vesicles (large EVs) derived from leiomyoma cell lines. Recipient myometrial cells were either left untreated (Control) or treated with large EVs isolated from leiomyoma cell lines under different conditions: large EVs from untreated leiomyoma cells (Control large EV F), large EVs from eicosapentaenoic acid-treated leiomyoma cells (EPA large EV F), and large EVs from docosahexaenoic acid-treated leiomyoma cells (DHA large EV F). Fibronectin protein expression was assessed by western blotting and normalized to the appropriate loading control. DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; lEVs = large extracellular vesicles.

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Uterine leiomyomas are benign smooth muscle tumors characterized by excessive ECM deposition, contributing to their fibrotic and stiff nature. Within a dense and stiff ECM environment typical of fibroids, long-distance diffusion of EVs is likely restricted. We hypothesize that EVs predominantly act in a paracrine fashion on immediate neighboring cells, such as mature smooth muscle cells and resident stem/progenitor cells or diffuse through localized microchannels (23).

Fibronectin, a major glycoprotein of the ECM, plays a central role in ECM turnover and fibrosis, with dysregulated expression implicated in fibroid development and progression (15, 18, 24–26). Other ECM components, including collagen types I and III, proteoglycans, and matricellular proteins, further contribute to tissue stiffening, abnormal mechanotransduction, and altered uterine architecture, leading to a spectrum of clinical manifestations such as menorrhagia, dysmenorrhea, pelvic pressure, and impaired fertility (23, 27).

Considering the present results, we hypothesize that EV cargo favors the transfer of bioactive molecules, including growth factors, cytokines, and matrix-modifying enzymes, which are critical in fibroid pathogenesis. The induction of fibrosis in adjacent healthy myometrium via EV paracrine signaling may therefore represent a mechanism underlying the frequent development of multiple leiomyomas. Although leiomyomas are monoclonal in origin, additional genetic or epigenetic events, unhealthy tissue milieu, or persistent external stimuli may contribute to the propagation of a fibrotic phenotype across the uterus.

Interestingly, EVs from leiomyoma cells pretreated with omega-3 fatty acids showed a reduction in fibronectin induction. Both EPA and DHA are known to integrate into cellular membranes, alter lipid composition, and modulate signaling pathways related to inflammation, lipid metabolism, and mechanotransduction (15).

These results suggest that omega-3 fatty acids may modify EV cargo or reduce the secretion of profibrotic factors, thereby decreasing their capacity to upregulate fibronectin in recipient cells. This emphasizes the potential therapeutic role of omega-3 fatty acids in managing uterine fibroids and other fibrotic disorders. In contrast, EVs derived from normal myometrial cells, with or without omega-3 fatty acid pretreatment, did not significantly affect fibronectin expression, supporting the notion that profibrotic signaling is context-dependent and specifically driven by leiomyoma-derived EVs.

Furthermore, mechanistic studies on EV cargo characterization and modification are warranted. Typically, lEVs are enriched in cytosolic proteins, ECM fragments, and signaling molecules, whereas sEVs predominantly contain microRNAs, tetraspanins, and membrane-associated signaling components. Proteomic studies of leiomyoma EVs have identified transforming growth factor-beta (TGF-β), connective tissue growth factor, platelet-derived growth factor, and profibrotic microRNAs such as miR-21, miR-29b, and miR-199a, all of which orchestrate ECM deposition and fibroblast activation (9, 10).

These molecules converge on canonical TGF-β/Smad pathways, promoting ECM gene transcription (e.g., FN1, COL1A1), while interacting with mechanotransduction pathways such as Rho/ROCK and YAP/TAZ signaling, reinforcing myofibroblastic differentiation and cytoskeletal remodeling in recipient cells. In leiomyoma cells, activin A further enhances these processes by activating Smad2/3 signaling and upregulating profibrotic genes, including collagen and fibronectin, as well as contractile markers such as α-SMA. This results in increased ECM deposition, enhanced contractility, and maintenance of the fibrotic, myofibroblast-like phenotype characteristic of leiomyoma tissue (28). A novel aspect of the present study is the observation that pretreatment of leiomyoma cells with omega-3 fatty acids markedly attenuates the profibrotic effects of their EVs. Omega-3 fatty acids modulate EV biogenesis, cargo sorting, and uptake, potentially reducing the loading of profibrotic proteins and microRNAs or altering vesicle lipid composition to decrease fusion efficiency. Additionally, EPA and DHA exert antiinflammatory effects through inhibition of NF-κB signaling, suppression of TGF-β1 expression, and upregulation of matrix metalloproteinases (MMPs), which degrade ECM components. Collectively, these effects reduce the fibrotic potential of leiomyoma EVs, limiting fibronectin deposition and potentially mitigating downstream consequences such as tissue stiffening, angiogenesis, and abnormal mechanotransduction.

The paracrine activity of leiomyoma-derived EVs may contribute to the creation of a profibrotic niche in the surrounding myometrium, facilitating fibroid expansion and altered uterine contractility. This EV-mediated signaling could help explain the frequent occurrence of multiple fibroids within a single uterus, with propagated fibrotic signals creating permissive conditions for new lesion development. Therapeutically, modulation of EV-mediated communication through dietary supplementation with omega-3 fatty acids or pharmacological inhibition of EV release and uptake represents a promising, noninvasive strategy to limit fibroid-associated fibrosis.

Despite the robustness of our methodology, which adhered to minimal information for studies of extracellular vesicles (MISEV) 2023 guidelines for EV isolation and characterization (including TEM, NTA, and western blotting for canonical markers), certain limitations should be acknowledged. In vitro models may not fully recapitulate the hormonal, vascular, and immune context of the uterus in vivo, and premenopausal hormonal milieu may influence EV composition and activity. Although fibronectin was chosen as a representative ECM marker, leiomyoma EVs contain heterogeneous cargo, including other profibrotic proteins, microRNAs, and bioactive lipids. Comprehensive proteomic, lipidomic, and transcriptomic analyses are required to fully elucidate molecular mechanisms underlying fibrotic activity and to identify specific biomarkers for disease progression or therapeutic targeting. Longitudinal in vivo studies are also warranted to assess the efficacy of omega-3 fatty acids in modulating EV-mediated fibrosis and to translate these findings into clinical strategies for fibroid management.

These observations are consistent with the growing concept that EVs serve not only as biomarkers but also as therapeutic targets and delivery systems in gynecological disorders (29, 30).

Our study presents some limitations that should be acknowledged. First, EV isolation was performed via differential ultracentrifugation; future studies should incorporate density gradient purification (e.g., iodixanol) to fully separate overlapping EV subpopulations. Second, we did not evaluate the genomic status (e.g., MED12 mutations) of our primary cell cultures. As leiomyomas exhibit significant cellular heterogeneity, isolated EVs may derive not only from monoclonal smooth muscle tumor cells but also from infiltrating stromal or immune cells. Additionally, future studies on larger cohorts should stratify EV release and content on the basis of specific clinical and histopathological features. Furthermore, we used fibronectin as a single surrogate marker for fibrotic remodeling. Future investigations must incorporate additional ECM markers (e.g., COL1A1, α-SMA) and evaluate profibrotic signaling pathways (e.g., TGF-β/Smad) to robustly confirm biological relevance. Finally, given the in vitro and exploratory nature of our functional data, conclusions regarding omega-3 fatty acids remain a proof-of-concept and require robust in vivo validation before any clinical application can be inferred.

CONCLUSION

In conclusion, our study demonstrates that leiomyoma-derived EVs appear to act as paracrine effectors, promoting fibronectin expression and ECM remodeling in recipient myometrial cells, contributing to uterine fibrosis and potentially facilitating fibroid growth. Extracellular vesicles from normal myometrial cells maintain tissue homeostasis, highlighting the pathological specificity of leiomyoma EV cargo. Importantly, treatment with omega-3 fatty acids (DHA and EPA) mitigates the profibrotic activity of leiomyoma EVs, representing a promising avenue for noninvasive therapeutic intervention. By elucidating the interplay between EV-mediated signaling, ECM remodeling, and mechanotransduction, these findings enhance understanding of fibroid biology and provide a framework for future translational research aimed at mitigating uterine fibrotic progression.

CRediT Authorship Contribution Statement

Stefania Greco: Writing – original draft, Methodology, Formal analysis, Data curation. **Rachele Agostini:** Writing – original draft, Methodology, Investigation, Data curation. **Michela Battistelli:** Writing – review & editing, Methodology, Data curation. **Giovanni Delli Carpini:** Writing – review & editing, Investigation. **Andrea Faragalli:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Abel Duménigo González:** Writing – review & editing, Methodology, Investigation. **Andrea Ciavattini:** Writing – review & editing, Supervision, Conceptualization. **Michele Guescini:** Writing – review & editing, Supervision, Conceptualization. **Pasquapina Ciarmela:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Interests

S.G. reports funding from Università Politecnica delle Marche Researcher Fellowship. R.A. has nothing to disclose. M.B. has nothing to disclose. G.D.C. has nothing to disclose. A.F. has nothing to disclose. A.D.G. has nothing to disclose. A.C. has nothing to disclose. M.G. has nothing to disclose. P.C. has nothing to disclose.

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