Sphingosine-1-phosphate receptor 3 is a non-hormonal target to counteract endometriosis-associated fibrosis

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Objective: To study the molecular mechanisms responsible for fibrosis in endometriosis by investigating whether the protein expression levels of sphingosine-1-phosphate receptor 3 (S1PR3), one of the five specific receptors of the bioactive sphingolipid sphingosine-1-phosphate (S1P), correlate with fibrosis extent in endometriotic lesions and which are the cellular mechanisms involved in this process.
Design: Case-control laboratory study and cultured endometriotic cells.
Setting: University research institute and university hospital.
Patient(s): A total of 33 women, with and without endometriosis, were included in the study.
Interventions(s): Endometriotic lesions were obtained from women with endometriosis (ovarian endometrioma, n = 8; deep infiltrating endometriosis, n = 15; [urological n = 5, gastrointestinal n = 6, and posterior n = 4]) and control endometrium from healthy women, n = 10, by means of laparoscopic and hysteroscopic surgery. The expression of S1PR3 was evaluated using immunohistochemistry and the extent of fibrosis was assessed using Masson's trichrome staining. Human-cultured epithelial endometriotic 12Z cells were used to evaluate the mechanisms involved in the profibrotic effect of S1PR3 activation.
Main Outcome Measure(s): The expression of S1PR3 in endometriotic lesions is positively correlated with endometriosis-associated fibrosis. In addition, S1P induced epithelial-mesenchymal transition (EMT) and fibrosis in epithelial endometriotic cells. Using RNA interference and pharmacological approaches, the profibrotic effect of S1P was shown to rely on S1PR3, thus unveiling the molecular mechanism implicated in the profibrotic action of the bioactive sphingolipid.
Result(s): The protein expression levels of S1PR3 were significantly augmented in the glandular sections of endometrioma and deep infiltrating endometriosis of different localizations with respect to the control endometrium and positively correlated with the extent of fibrosis. Sphingosine-1-phosphate was shown to have a crucial role in the onset of fibrosis in epithelial endometriotic cells, stimulating the expression of EMT and fibrotic markers. Genetic approaches have highlighted that S1PR3 mediates the fibrotic effect of S1P. Downstream of S1PR3, ezrin and extracellular-signal-regulated kinases 1 and 2 signaling were found to be critically implicated in the EMT and fibrosis elicited by S1P.
Conclusion(s): Sphingosine-1-phosphate receptor 3 may represent a possible innovative pharmacological target for endometriosis. (Fertil Steril® 2024;121:631-41. ©2023 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Sphingosine-1-phosphate receptor 3, endometriosis non-hormonal target, fibrosis, ezrin, ERK1/2

Endometriosis is a severe inflammatory and sex-steroid hormone-dependent gynecologic illness that affects 6%–10% of reproductive-age women and is characterized by the presence of endometrial glands and stroma outside of the uterine cavity (1). Menstruation-related discomfort (dysmenorrhea, dyspareunia, dysuria, and dyschezia), which has a significant impact on women's quality of life, and infertility are the most typical endometriosis symptoms (2–4).

Three phenotypes of endometriotic lesions have been identified: deep infiltrating endometriosis (DIE), ovarian endometrioma (OMA), and superficial peritoneal lesions. Although DIE and OMA share similar pathogenetic pathways, distinct characteristics account for the varied symptomatology (3, 4).

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Endometriotic lesions are usually distinguished by endometrial cell proliferation, invasion, and oxidative stress, in addition to inflammatory and neuro-angiogenetic traits (5). Moreover, a fibrotic phenotype, described at varying degrees depending on the localization of the lesion, is one of the causes of persistent pain and infertility. Fibrosis, characterized by the excessive accumulation of extracellular matrix (ECM) components, leads to permanent scarring and loss of normal architecture and tissue function. The main cells responsible for the remodeling and maturation of the scar tissue are myofibroblasts, which are cells derived from activated fibroblasts (fibroblast-to-myofibroblast transdifferentiation) or from the transdifferentiation of epithelial (epithelial-mesenchymal transition [EMT]) or endothelial (endothelial-mesenchymal transition) cells. The presence of fibrotic tissue inside and surrounding the endometriotic ovarian and peritoneal lesions is the main reason for pelvic discomfort in endometriosis brought about by tissue adhesions, scarring, and anatomic distortions (2, 6). However, the molecular mechanisms underlying the development of the fibrotic phenotype in endometriotic lesions remain to be fully elucidated.

Sphingosine 1-phosphate (S1P) is a bioactive pleiotropic sphingolipid (7), recently demonstrated to be crucial for fibrosis development (2, 8). Sphingosine 1-phosphate metabolism and signaling are complex and finely regulated (9, 10). Two sphingosine kinase isoforms, SK1 and SK2, synthesize S1P, which in turn can be reversibly dephosphorylated by specific and aspecific phosphatases or irreversibly degraded by S1P lyase (11). After being released into the extracellular environment by the selective transporter spinster homologue 2 as well as by other unspecific transporters (12), S1P acts as a ligand for specific G protein-coupled receptors named S1PR (S1PR 1–5), which are responsible for most of its actions (10).

Sphingosine 1-phosphate metabolism is deeply influenced by cross-talk with multiple profibrotic mediators such as transforming growth factor beta (TGF-β) and activin (13, 14). The fine balance between the synthesis and the catabolism of the sphingolipid is crucial for the final biological effect evoked by S1P. In a lung injury model, SK1 deficiency protects from fibrosis, whereas S1P lyase deficiency enhances the fibrogenetic process (15).

Sphingosine 1-phosphate metabolism is altered in endometriotic lesions (16, 17), and the concentration of the bioactive sphingolipid has been found recently to increase in the peritoneal fluid of patients with OMA with respect to controls (18). We recently demonstrated that endometriosis is characterized typically by increased messenger RNA (mRNA) levels of S1PR1, S1PR3, and S1PR5 compared with healthy endometrium (16). Moreover, S1P mediates TGF-β1-induced fibrosis via S1PR2 and S1PR3 in uterine adenocarcinoma cells (16). In agreement with the concept that S1P plays a key role in the pathogenesis of endometriosis, the pharmacological blockade of SK1 suppressed the development of endometriotic lesions in mice (19).

In this study, we analyzed the expression of the receptor S1PR3 at the protein level in OMA and DIE (urological, intestinal, and posterior) and its possible correlation with the extent of fibrosis. Moreover, the involvement of S1PR3 signaling in the induction of fibrosis and EMT has been investigated in endometriotic epithelial cells.

MATERIALS AND METHODS

Materials

The immortalized human endometriotic epithelial 12Z cell line and the Applied Cell Extracellular Matrix were obtained from Applied Biological Materials Inc. (Richmond, British Columbia, Canada). Bradford's reagent (Blue Coomassie G250), Tris-glycine-sodium dodecyl sulfate (SDS) buffer, polyvinylidene fluoride membranes for the Trans-Blot Turbo instrument, EveryBlot Blocking Buffer, and chemiluminescent reagents were purchased from Bio-Rad (Hercules, CA, USA). All biochemicals, Dulbecco's modified Eagle's medium (DMEM), Nutrient Mixture F-12 Ham (F12), fetal bovine serum (FBS), protease inhibitor cocktail, phosphatase inhibitor cocktail 3, bovine serum albumin, TRI-Reagent, 30% acrylamide and bis-acrylamide solution, the scramble and specific small interfering RNAs (siRNAs) for S1PR1, S1PR2, S1PR3, S1PR4, S1PR5, the ReBlot Plus Strong Antibody Stripping Solution, the specific ezrin inhibitor NSC668394, the specific extracellular-signal-regulated kinases 1 and 2 (ERK1/2) inhibitor U0126, and the anti-αSMA mouse primary antibody were purchased from Merck Life Sciences (Burlington, MA, USA). The mouse primary antibodies against α-tubulin, anti-vimentin, anti-N-cadherin, anti-SNAI-1, and the horseradish peroxidase-conjugated anti-mouse, anti-goat, as well as anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The goat anti-transgelin primary antibody was purchased from Everest (Bicester, UK). Primary rabbit antibodies against the phosphorylated form of ERK 1 and 2 as well as ezrin-radixin-moesin (ERM) were purchased from Cell Signalling (Danvers, MA, USA). The rabbit anti-S1PR3 primary antibody was obtained from Abcam (Cambridge, UK). TaqMan Universal Master Mix II for Real Time PCR, TaqMan gene expression assays (S1PR1: Hs01922614_s1; S1PR2: Hs_01003373_M1; S1PR3: Hs00245464_s1; S1PR4: Hs02330084_s1; S1PR5: Hs00928195_s1), and RNAiMAX lipofectamine, as well as the "UltraVision LP Detection System" were purchased from Thermo Fisher (Waltham, MA, USA).

Tissue Samples

Tissue samples were obtained from patients affected by endometriosis and undergoing laparoscopic surgery at the University Hospital of Careggi (Florence, Italy). Endometriosis samples were categorized as follows: OMA (n = 8) or DIE (total n = 15; urological n = 5, gastrointestinal n = 6, and posterior n = 4). The stage of the disease, reflecting its severity, was assigned after the AFSr classification system. Control endometrial samples were acquired using diagnostic hysteroscopy procedures, which were performed on a total of 10 women during the proliferative phase of the uterine cycle. Clinical and imaging investigations were performed to exclude a diagnosis of endometriosis or other uterine disorders in controls. All the samples were histologically characterized. All hormonal treatments were suspended for at least 3 months before surgery. The endometrial cycle phase was confirmed by histologic analysis of endometrial biopsies. There were no differences in age, pregnancy, and parity

between the study and control groups. The Institutional Review Board approved the study protocol (number 13,742, March 11, 2019), and all patients signed informed written consent.

Immunohistochemistry

Tissue samples were fixed with formalin and paraffin embedded. Serial 3 μm sections were obtained from each block, and subsequent slides were stained for immunohistochemistry (IHC) analysis of S1PR3 (1:75; Abcam [Cambridge, UK]) and Masson Trichrome staining. Routine deparaffinization and rehydration procedures were performed. For antigen retrieval, the slides were heated at 95–97 °C in a citrate buffer (pH 6.0) for a total of 20 minutes and cooled to room temperature (RT). The slides were then incubated with the primary antibodies overnight at 4 °C. The "UltraVision LP Detection System HRP Polymer and DAB Plus Cromogen" (Thermo Fisher) kit was used to detect the antigen-antibody complexes following the manufacturer's instructions: tissue slices were covered with the "Primary Antibody Enhancer" for 10 minutes at RT and incubated for 15 minutes at RT with the secondary antibody conjugated to the horseradish peroxidase. The bound antibody complexes were stained for 3.5 minutes for microscopic examination with diaminobenzidine and mounted. Images were obtained using a Nikon DS F12 CCD camera connected to a Nikon Eclipse E200 light microscope. The quantification of the S1PR3 positive area was morphometrically assessed within the glandular area on digitized images (at least 15 randomly selected images) acquired with a $\times$10 objective using the threshold tool of ImageJ software (National Institutes of Health, USA) (20). The results are expressed as the ratio between the S1PR3 positive area and the total glandular area considered in the analysis.

Masson's Trichrome Stain

Tissue slices were analyzed for fibrosis extent using Masson's trichrome staining. Specifically, the tissue slices were deparaffinized, rehydrated, and fixed by overnight incubation with Bouin's solution. The next day, washings were performed in running and distilled water, interspersed with incubation of the slides with the dyes of the Trichrome stain (Masson) kit (Merck Life Sciences), following the manufacturer's instructions: hematoxylin, which stains cell nuclei black; acid fuchsine, which highlights the cytoplasm in red; and aniline blue, which colors collagen fibers in blue. A solution containing phosphomolybdic acid and phosphotungstic acid, which act as mordants for aniline blue, was also used. Finally, the tissue slices were dehydrated using passages in the increasing series of alcohols, washed in xylene, covered with the coverslip, and stored at RT. Images were obtained using a Nikon DS F12 CCD camera connected to a Nikon Eclipse E200 light microscope. The quantification of collagen-positive structures was morphometrically assessed in 10 randomly selected fields acquired with a $\times$4 objective using the threshold tool of ImageJ software. The results are expressed as the ratio between the collagen-positive area and the total area considered in the analysis.

Cell Culture and Treatment

Human endometriotic epithelial 12Z cells were grown in a 1:1 mixture of DMEM:F12, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2. For the experiments, cells were seeded, and the next day they were serum-starved overnight in a medium without serum supplemented with 1 mg/mL fatty acid-free bovine serum albumin. To enhance cellular adhesion, the plates were coated with an applied cell ECM before use. When requested, cells were preincubated with pharmacological inhibitors 1 hour before S1P treatment.

Cell Transfection

Endometriotic epithelial cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX, according to the manufacturer's instructions as described in (21–23). Briefly, Lipofectamine RNAiMAX was incubated with siRNAs in DMEM:F12 without serum and antibiotics at RT for 20 min and then added to cells to a final concentration of 50 nM, in DMEM:F12 containing FBS. After 30 h cells were serum-starved overnight and used for experiments 48 h after the beginning of transfection. The efficacy of specific gene knockdown was evaluated using real-time reverse-transcription polymerase chain reaction.

Real-Time Polymerase Chain Reaction

Total RNA extracted from cells (1 μg) with the use of TRI-Reagent was reverse transcribed with the high-capacity copy DNA reverse-transcription kit (Thermo Fisher) following the manufacturer's protocol as described previously (24, 25). The quantification of target gene mRNAs was performed in triplicate with the use of real-time PCR using TaqMan gene expression assays and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Target sequences were simultaneously amplified with the housekeeping gene β-actin. Relative quantification of mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method (26).

Western Blotting

Cells were collected and lysed for 30 minutes at 4 °C in a buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 6 mM ethylene glycol tetraacetic acid, 15 mM $Na_4P_2O_7$, 20 mM NaF, 1% nonidet, and a protease inhibitor cocktail. Then, lysates were centrifuged at 10,000 g for 15 minutes at 4 °C, and 10 μg of protein were used to perform SDS and polyacrylamide gel electrophoresis as well as Western blotting (27, 28). Polyvinylidene fluoride membranes were incubated overnight with the primary antibodies at 4 °C and then with specific secondary antibodies for 1 hour at RT. The binding of the antibodies to the specific proteins has been detected using chemiluminescence employing the Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was performed busing ImageJ software. α-tubulin expression analysis was performed to ensure equal protein loading. The intensity of the band corresponding to the protein of interest was normalized to that of

α-tubulin and reported as a value relating to the intensity of the band corresponding to the control, set equal to 1.

Statistical Analysis

Data were analyzed by Student's *t*-test, one-way ANOVA, two-way ANOVA, followed by a Bonferroni post hoc test, and Pearson's test. Graphical representations were made using the GraphPad Prism 10.0 software.

RESULTS

The protein expression level of S1PR3, previously demonstrated to be increased at the mRNA level both in OMA and DIE (16), was quantified using IHC in OMA (n = 8) and DIE of different localizations such as urological (n = 5), gastrointestinal (n = 6), and posterior (n = 4), as well as in the endometrium of healthy women (control, n = 10). Representative images, shown in Figure 1 (*left panel*), depict that S1PR3 staining was detected in both endometriotic epithelial and stromal cells, mostly in the cytoplasm and membranes. Interestingly, the levels of S1PR3 were significantly augmented in all the analyzed glandular sections of endometriotic lesions with respect to the control endometrium, in favor of increased signaling of the bioactive sphingolipid S1P through S1PR3 in endometriosis (Fig. 1, *left panel*).

In addition, Masson's trichrome staining was performed in the same samples, and the representative images reported in Figure 1 (*right panel*) highlight that the extent of fibrosis, quantified using blue-stained collagen fibers, is almost absent in the control endometrium, whereas it is dramatically augmented in all the analyzed endometriotic lesions. Linear regression analyses indicated that the staining levels of S1PR3 positively correlated with the extent of fibrosis (Pearson correlation coefficients: r = 0.89, $^{*}P<.01$ for OMA; r = 0.72, $^{*}P<.01$ for DIE), demonstrating the occurrence of a strong correlation between S1PR3 and endometriosis-associated fibrosis.

To investigate the involvement of S1P in the development of endometriosis-associated fibrosis, *in vitro* studies were performed in endometriotic epithelial 12Z cells. In particular, the ability of S1P to induce fibrosis and EMT was assessed using Western blot analysis performed in cells treated or not with 1 μM S1P for 48 hours. Results shown in Figure 2 proved that S1P induced a strong increase in the expression of the EMT markers N-cadherin, vimentin, SNAI-1, and the fibrotic marker transgelin, demonstrating a crucial role for the bioactive sphingolipid in the fibrotic process in endometriotic cells. To get insight into the molecular mechanisms of S1P-induced EMT and fibrosis, we studied whether the bioactive sphingolipid was able to activate ERM or ERK 1 and 2, known to be involved in fibrosis and EMT as well as other biological responses (29–31). As reported in Figure 3A, S1P rapidly and transiently activated ERM as well as ERK 1 and 2 by approximately 4- and 3-fold, respectively, at the peak of activation (1 minute challenge with the sphingolipid).

We next examined whether S1P-induced activation of ERM as well as ERK 1 and 2 was mediated by S1PR. For this purpose, all the S1PR isoforms were efficiently silenced by RNA interference with specific siRNAs (Supplemental Fig. 1, available online). Of note, only the selective knockdown of S1PR3 significantly reduced the activation of ERM as well as ERK 1 and 2 elicited by S1P. Conversely, the down-regulation of S1PR1, S1PR2, S1PR4, and S1PR5 did not affect the S1P-induced action (Fig. 3B). Next, to investigate whether these signaling pathways activated by S1P through S1PR3 are implicated in the profibrotic effect of the bioactive sphingolipid, endometriotic cells were preincubated with NSC668394 (2.5 μM) or U0126 (5 μM), which specifically inhibits ezrin as well as ERK 1 and 2, respectively, before the S1P challenge. As shown in Figure 4A, the blockade of ERK 1 and 2 or ezrin activation strongly reduced the increase of EMT and fibrosis marker expression brought about by S1P in endometriotic cells. These data suggest a crucial role for the S1PR3-dependent activation of ERK 1 and 2 and ezrin in the induction of EMT and fibrosis triggered by S1P. In accordance, Western blot analysis shown in Figure 4B demonstrated that the down-regulation of S1PR3 by RNA interference fully abolished the fibrotic effect of S1P in endometriotic cells, further highlighting the pivotal role of this receptor isoform in the detrimental effect elicited by S1P in endometriosis.

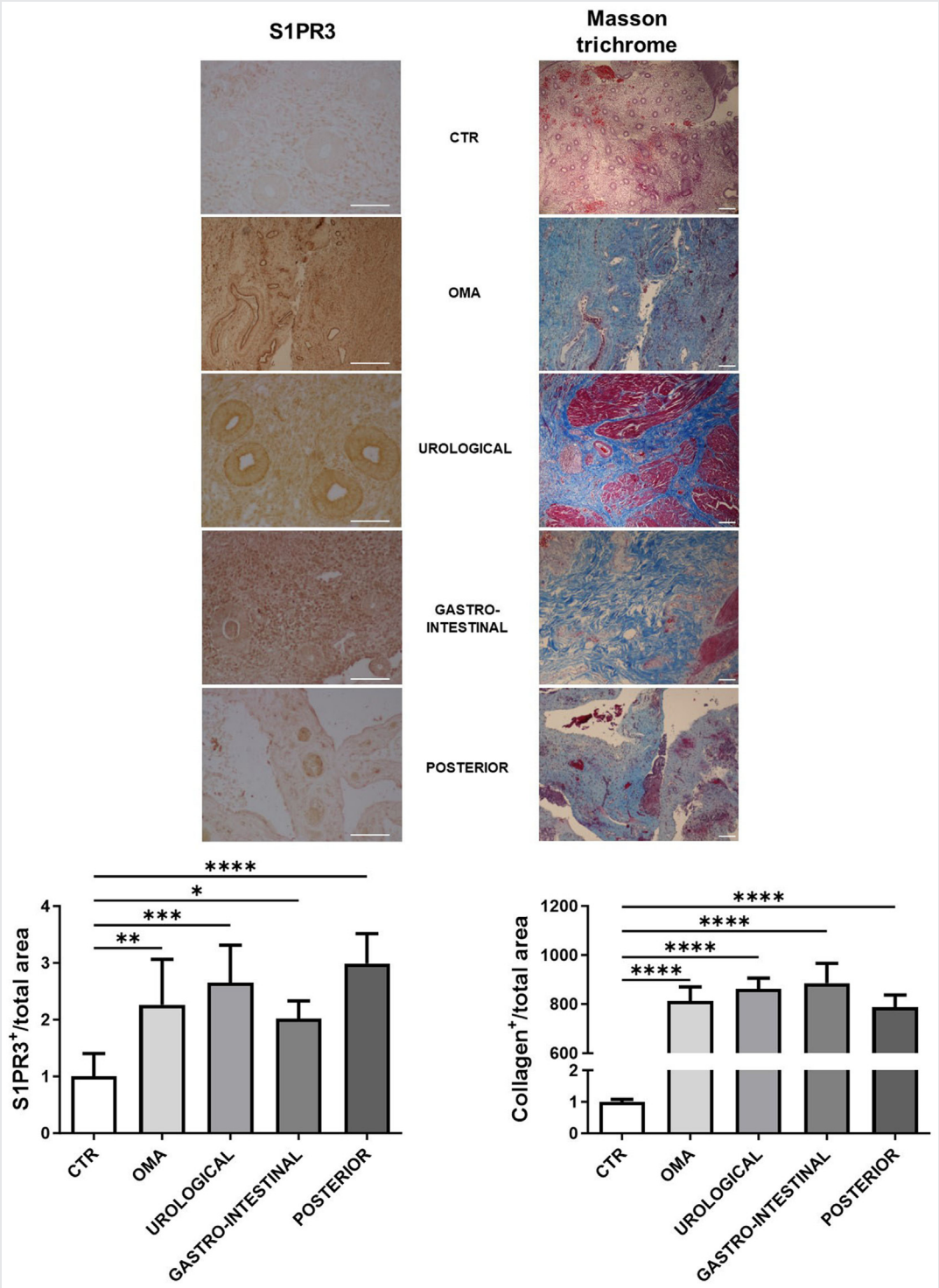
DISCUSSION

Fibrosis and EMT are emerging as key processes in the pathophysiology of endometriosis, being involved in lesion formation, pain, and infertility (3). The available current interventions for the disease are unsatisfactory, relying on surgical removal of the lesions and hormonal therapies with high symptom relapse and important collateral effects, respectively (1).

The findings reported in the present study provide further support for the crucial involvement of S1P signaling in the development of endometriosis-associated fibrosis. Indeed, for the first time, a solid link between fibrosis and the protein expression levels of the specific S1P receptor S1PR3 has been shown: the immunostaining levels of the receptor positively correlated with endometriosis-associated fibrosis extent evaluated by Masson's trichrome staining in endometriotic lesions of different localizations (OMA, urological, gastrointestinal, and posterior), suggesting a strong association of S1P signaling with the fibrotic trait of the disease. Moreover, the crucial role of S1PR3 in fibrogenesis, via the downstream activation of ERK 1 and 2, as well as ezrin, in human epithelial endometriotic cells has been demonstrated here. Indeed, Western blot analysis demonstrated that the down-regulation of S1PR3 by RNA interference fully abolished the profibrotic effect of S1P and strongly diminished the phosphorylation of ERK 1 and 2 as well as ERM-induced by the sphingolipid in endometriotic cells. Finally, the pharmacological blockade of ezrin as well as ERK 1 and 2 phosphorylation induced by S1PR3 activation abolished the S1P-induced increase of the EMT and fibrosis markers, unveiling the molecular mechanism by which this receptor isoform mediates the detrimental effect elicited by the bioactive sphingolipid in endometriosis.

Numerous studies have established previously the typical occurrence of fibrosis and myofibroblast activation at the level of endometriotic lesions as well as their significance in

FIGURE 1



Sphingosine-1-phosphate receptor 3 (S1PR3) levels as well as fibrosis extent are augmented in endometriotic lesions. Immunohistochemical analysis of S1PR3 expression and Masson's trichrome stain in endometriotic lesions (n = 8 OMA, n = 5 urological, n = 6 gastrointestinal, n = 4 posterior) and the endometrium of healthy women (n = 10 CTR). Magnification: ×10 for S1PR3, scale bar: 200 μm; ×4 for Masson's trichrome stain, scale bar: 100 μm. Results illustrated in the left histogram represent the ratio (mean ± SD) between the S1PR3 positive area and the total glandular area considered in the analysis and are reported as a fold change over the endometrium of healthy women (set as 1). Results illustrated in the right histogram represent the ratio (mean ± SD) between the collagen-positive area and the total area considered in the analysis and are reported as fold change over the endometrium of healthy women (set as 1). Statistical analyses were performed using one-way ANOVA (*P<.05; **P<.01; ***P<.001; ****P<.0001). CTR = control; OMA = ovarian endometrioma.

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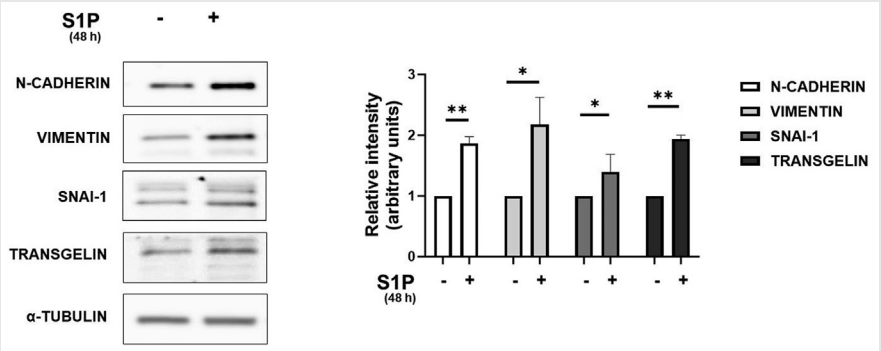
FIGURE 2



Sphingosine-1-phosphate (S1P) induces epithelial-mesenchymal transition and fibrosis in endometriotic epithelial cells. Endometriotic epithelial cells were treated with 1 μM S1P for 48 hours. Protein lysates were analyzed using SDS-PAGE electrophoresis and Western blotting, using specific anti-N-cadherin, anti-vimentin, anti-SNAI-1, anti-transgelin, and anti-α-tubulin antibodies. The histogram represents the densitometric analysis of two independent experiments. Data are the mean ± SD and are reported as protein expression normalized to α-tubulin, fold change over control (set as 1). The effect of S1P is statistically significant (*t*-test, **P*<.05, ***P*<.01). SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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the pathophysiology of the condition (6, 32, 33): consequent pelvic adhesions involving different tissue districts (bladder, ureter, and colon) can cause dislocation of the implicated organs and pain (6).

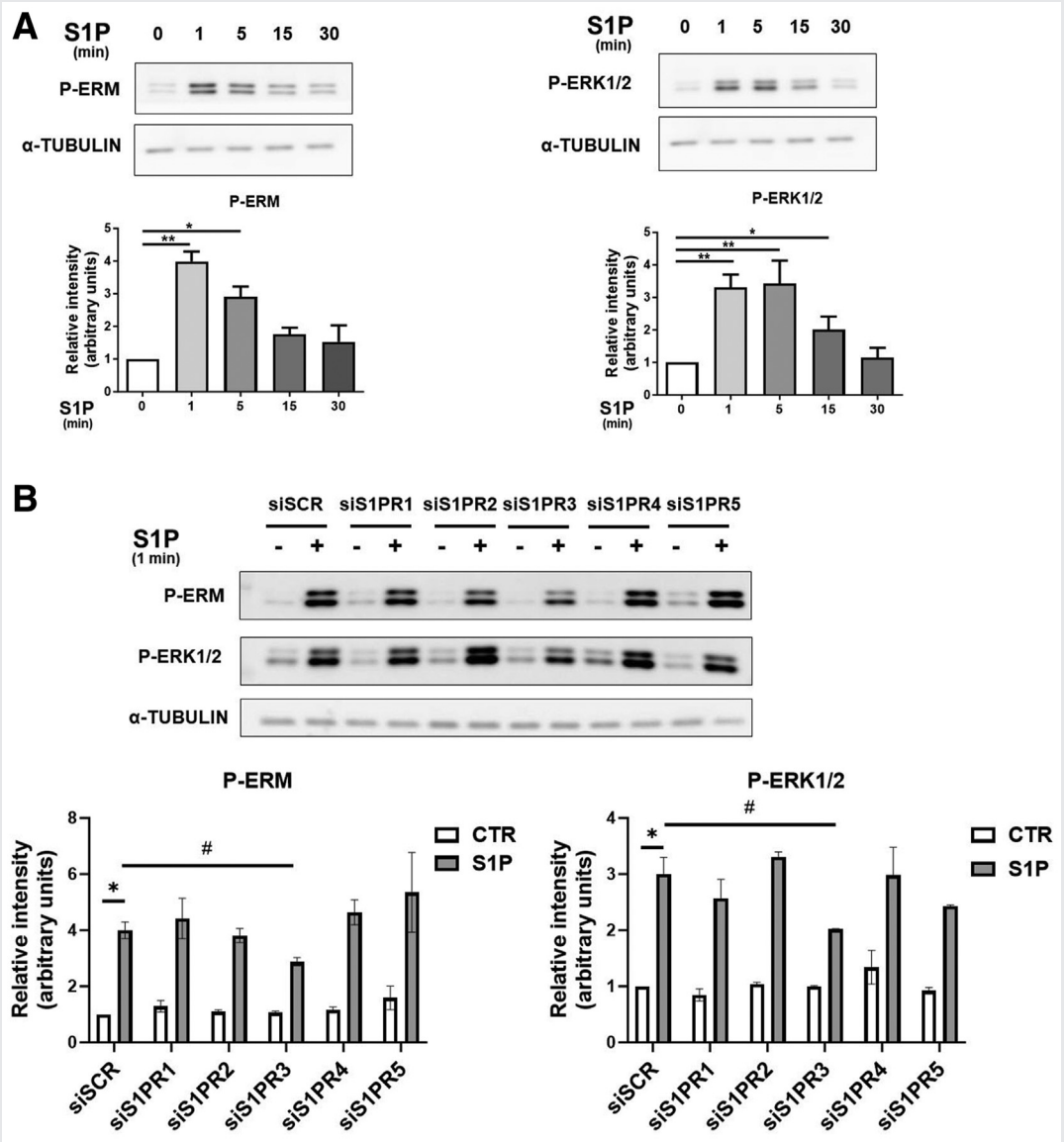
Solid literature evidence supports the view that inflammatory mediators, such as TGF-β, are crucially involved in endometriosis-associated fibrogenesis, not only stimulating the deposition of ECM but also mediating EMT (6, 34). Multiple studies have shown that S1P is involved in EMT via the regulation of TGF-β1 signaling, thus contributing to fibrosis (8, 35, 36). Sphingosine-1-phosphate has been demonstrated also to modulate the levels of matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9, regulating ECM remodeling (37, 38). The present study focuses on the role of S1PR3 and S1P signaling in the modulation of fibrosis, highlighting that the bioactive sphingolipid S1P induces EMT in human epithelial endometriotic cells, causing a significant increase in the markers N-cadherin, vimentin, SNAI-1, and transgelin. However, taking into consideration the pivotal role of fibroblast-to-myofibroblast transdifferentiation in fibrogenesis, future studies will be required to deeply investigate the role of S1PR3 and its signaling in the stromal endometriotic compartment.

The signaling of the bioactive sphingolipid is involved in the etiopathogenesis of multiple disease states, such as cancer, inflammation, and fibrosis (8, 11, 39, 40). Of note, the levels of S1P are augmented in the peritoneal fluid of women with endometriosis (18). In addition, the signaling and metabolism of the bioactive sphingolipid are deeply dysregulated at the level of endometriotic lesions (16, 17). Moreover, we recently showed that S1P induces a reactive oxygen species-mediated proinflammatory response in human endometrial stromal cells (41), further suggesting a crucial role of S1P in the endometriosis-associated inflammatory scenario.

Notably, the S1PR3 isoform has been crucially linked to the development of fibrosis in different tissues, such as skeletal muscle, lung, kidney, and heart (42–46). The transdifferentiation of myoblasts into myofibroblasts triggered by TGF-β1 is mediated by SK1 and S1PR3 signaling in skeletal muscle myoblasts. Increased levels of the cytokine are responsible for the up-regulation of SK1 and S1PR3, which become the most expressed receptors, causing a shift in signaling from myogenic to fibrotic phenotypes (44). Sphingosine-1-phosphate receptor 3 was shown to mediate the ability of TGF-β1 to induce fibrosis and EMT markers in a model of uterine adenocarcinoma cells (16). In a SK1 transgenic mouse model, S1PR3 deletion was shown to inhibit Smad-associated cardiac fibrosis (42) and, in normal lung fibroblasts, the activation of S1PR2 and S1PR3 was demonstrated to induce the synthesis of ECM (47).

Extracellular-signal-regulated kinases 1 and 2 crucially operate in intracellular signaling pathways by phosphorylating specific serine and threonine residues of target molecules in response to a broad array of stimuli (48). Although the effects of ERK 1 and 2 activations are strictly dependent on cell type and the specific stimulus, they typically entail the regulation of cellular proliferation, survival, and differentiation, as well as carcinogenesis and fibrosis (31, 49). Activation of ERK 1 and 2 was reported to mediate TGFβ-2 and HGF-induced EMT in human epithelial lens cells and a mouse model of liver carcinogenesis (50). Notably, ERK 1 and 2 activity has been found to be abnormally regulated in the endometrial tissue of women with endometriosis (51, 52). Ezrin belongs to the ERM family of proteins that, localized beneath the plasma membrane of cellular protrusions (53), act as linkers between the plasma membrane and the actin cytoskeleton and are involved in cell adhesion and membrane ruffling (54). Eutopic and ectopic endometrial tissues from women affected by endometriosis have been demonstrated to be characterized by higher levels of ezrin and phospho-ezrin using IHC and Western blot analysis (55). Recently, ezrin has been shown to regulate cell migration and invasion in TGF-β1-induced EMT in

FIGURE 3



Role of sphingosine-1-phosphate receptor (S1PR) in sphingosine-1-phosphate (S1P)-induced activation of ezrin-radixin-moesin (ERM) as well as extracellular-signal-regulated kinases 1 and 2 (ERK1/2). (**A**) Endometriotic cells were overnight serum-starved and then treated with 1 μM S1P for the indicated time intervals (1–30 minutes). Protein lysates were analyzed using SDS-PAGE electrophoresis and Western blotting, using specific anti-P-ERM, anti-P-ERK 1 and 2, and anti-α-tubulin antibodies. The histograms represent the densitometric analysis of two independent experiments. Data are the mean ± SD and are reported as band intensity normalized to the expression of α-tubulin, fold change over control (set as 1). Statistical analysis was performed with one-way ANOVA (*P<.05; **P<.01). (**B**) Endometriotic cells were transfected for 30 hours with a nonspecific siRNA (scrambled, SCR) or with specific siRNAs for S1PR1, S1PR2, S1PR3, S1PR4, or S1PR5 before being treated with 1 μM S1P for 1 minute. Protein lysates were analyzed using SDS-PAGE electrophoresis and Western blotting using specific anti-P-ERM, anti-P-ERK 1 and 2, and anti-α-tubulin antibodies. The histograms represent the densitometric analysis of two independent experiments. Data are the mean ± SD and are reported as band intensity normalized to the expression of α-tubulin, fold change over control (set as 1). Sphingosine-1-phosphate activated P-ERM and ERK 1 and 2 in a statistically significant manner (*P<.05). The effect of S1PR3 down-regulation on S1P-induced ERK 1 and 2 as well as ERM activation is statistically significant by two-way ANOVA, followed by Bonferroni's post hoc test (#P<.05). SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA = small interfering RNA.

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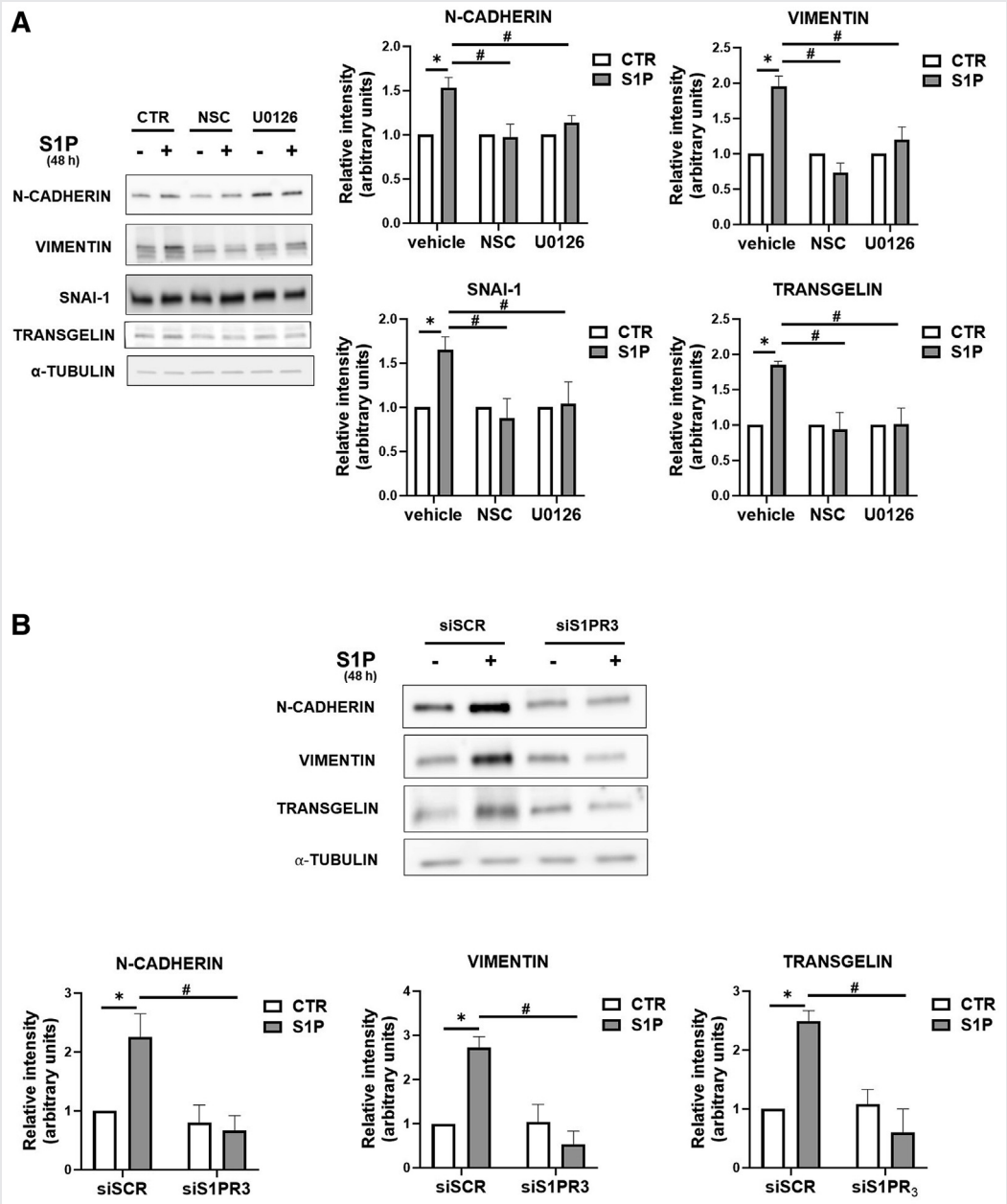
human alveolar epithelial cells (29). In the present study, we showed that S1P, via S1PR3, was able to rapidly and transiently increase the activation of ERK 1 and 2 as well as ezrin, and when their activation was pharmacologically blocked, the sphingolipid was unable to augment the expression levels of the fibrotic and EMT markers.

Taken together, these data expand our knowledge of the molecular mechanisms responsible for endometriosis

FIGURE 4



Sphingosine-1-phosphate receptor 3 (S1PR3) mediates sphingosine-1-phosphate (S1P)-induced ezrin-radixin-moesin (ERM) as well as extracellular-signal-regulated kinases 1 and 2 (ERK1/2). (**A**) Epithelial endometriotic cells were preincubated for 1 h in the presence or absence of NSC668394 (2.5 μM, NSC) and U0126 (5 μM), before being stimulated for 48 hours with 1 μM S1P. Protein lysates were analyzed using SDS-PAGE electrophoresis and Western blotting using specific anti-N-cadherin, anti-vimentin, anti-SNAI-1, anti-transgelin, and anti-α-tubulin antibodies. The histograms represent the densitometric analysis of two independent experiments. Data are the mean ± SD and are reported as protein expression normalized to α-tubulin, fold change over control (set as 1). Sphingosine-1-phosphate significantly induces the expression of EMT and fibrotic markers (*P<.05). The pharmacological inhibition of phosphorylation of ERK 1 and 2 by U0126 or ezrin by NSC668394 significantly affected the fibrotic effect of S1P using a two-way ANOVA followed by Bonferroni's post hoc test (#P<.05). (**B**) Endometriotic cells were transfected for 30 hours with a nonspecific siRNA (scrambled, SCR) or with siRNA specific for S1PR3 before being treated with 1 μM S1P for 48 hours. Protein lysates were analyzed using SDS-PAGE electrophoresis and Western blotting using specific anti-vimentin, anti-N-cadherin, anti-transgelin, and anti-α-tubulin antibodies. The histograms represent the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to α-tubulin, fold change over control (set as 1). Sphingosine-1-phosphate elicited the expression of vimentin, N-cadherin, and transgelin in a statistically significant manner (*P<.05). The effect of S1PR3 down-regulation on S1P-induced expression of EMT and fibrotic markers is statistically significant using a two-way ANOVA, followed by Bonferroni's post hoc test (#P<.05). EMT = epithelial-mesenchymal transition; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA = small interfering RNA.

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pathogenesis and reinforce the rationale for the exploitation of S1P signaling as an innovative therapeutic target for its treatment. Indeed, the signaling axis of the bioactive sphingolipid has been already employed in approaches based on S1PR modulators, such as fingolimod and ozanimod, for the treatment of multiple sclerosis and other immune syndromes (56, 57).

CONCLUSIONS

The present data showed for the first time a positive correlation between the extent of fibrosis and S1PR3 immunostaining levels in different types of endometriotic lesions (OMA and DIE). The crucial role of S1PR3 and its dependent activation of ERK 1 and 2, as well as ezrin signaling, in the profibrotic effect of S1P was demonstrated in human epithelial endometriotic cells. Therefore, S1PR3 may be proposed as a possible target to counteract the inflammation-associated fibrotic trait of endometriosis.

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CRediT Authorship Contribution Statement

Caterina Bernacchioni: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Margherita Rossi:** Writing – original draft, Investigation, Formal analysis. **Valentina Vannuzzi:** Investigation, Formal analysis. **Matteo Prisinzano:** Investigation, Formal analysis. **Isabelle Seidita:** Investigation, Formal analysis. **Maryam Raeispour:** Investigation, Formal analysis. **Angela Muccilli:** Investigation, Data curation. **Francesca Castiglione:** Data curation. **Paola Bruni:** Writing – review & editing, Funding acquisition, Conceptualization. **Felice Petraglia:** Writing – review & editing, Funding acquisition, Conceptualization. **Chiara Donati:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of Interests

C.B. has nothing to disclose. M. Rossi has nothing to disclose. V.V. has nothing to disclose. M.P. has nothing to disclose. I.S. has nothing to disclose. M. Raeispour has nothing to disclose. A.M. has nothing to disclose. F.C. has nothing to disclose. P.B. has nothing to disclose. F.P. has nothing to disclose. C.D. has nothing to disclose.

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El receptor 3 de la esfingosina-1-fosfato es una diana no hormonal para contrarrestar la fibrosis asociada a la endometriosis.

Objetivo: Estudiar los mecanismos moleculares responsables de la fibrosis en la endometriosis investigando si la expresión proteica del receptor 3 de esfingosina-1-fosfato (S1PR3), uno de los cinco receptores específicos del esfingolípido bioactivo esfingosina-1-fosfato (S1P), se correlacionan con la extensión de la fibrosis en las lesiones endometriosis.
Fosfato (S1P), se correlacionan con la extensión de la fibrosis en las lesiones endometriósicas y cuáles son los mecanismos celulares implicados en este proceso.

Diseño: Estudio de laboratorio de casos y controles y cultivo de células endometriósicas.

Entorno: Instituto universitario de investigación y hospital universitario.

Paciente(s): Un total de 33 mujeres, con y sin endometriosis, fueron incluidas en el estudio.

Intervenciones: Se obtuvieron lesiones endometriósicas de mujeres con endometriosis (endometrioma ovárico, n = 8; endometriosis infiltrante profunda, n = 15; urológica n = 5, gastrointestinal n = 6, y posterior n = 4]) y endometrio de control de mujeres sanas, n =10, mediante cirugía laparoscópica e histeroscópica. La expresión de S1PR3 se evaluó mediante inmunohistoquímica y la extensión de la fibrosis se evaluó mediante la tinción tricrómica de Masson. Se utilizaron células endometriósicas epiteliales 12Z cultivadas en seres humanos para evaluar los mecanismos implicados en la fibrosis endometriósica.
Para evaluar los mecanismos implicados en el efecto profibrótico de la activación de S1PR3.

Medida(s) principal(es) de resultado: La expresión de S1PR3 en lesiones endometriósicas se correlaciona positivamente con la fibrosis asociada a endometriosis. Además, la S1P induce la transición epitelio-mesénquima (EMT) y la fibrosis en células endometriósicas epiteliales. Mediante ARN y farmacológicos, se demostró que el efecto profibrótico de la S1P depende de la S1PR3, desvelando así el mecanismo molecular implicado en la profibrosis.

Resultados: Los niveles de expresión proteica de S1PR3 aumentaron significativamente en las secciones glandulares de endometriomas y endometriosis infiltrante profunda de diferentes localizaciones con respecto al endometrio de control y se correlacionaron positivamente con la extensión de la fibrosis.
Se demostró que la esfingosina-1-fosfato desempeña un papel crucial en el inicio de la fibrosis en las células endometriósicas epiteliales, estimulando la expresión de marcadores EMT y fibróticos. Los enfoques genéticos han puesto de relieve que S1PR3 media el efecto fibrótico de S1P.
En la corriente descendente de S1PR3, la ezrin y la señalización de las quinasas reguladas por señales extracelulares 1 y 2 resultaron estar críticamente implicadas en la EMT y la fibrosis provocadas por S1P.

Conclusiones: El receptor 3 de esfingosina-1-fosfato puede representar una posible diana farmacológica innovadora para la endometriosis.