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Role of Sphingosine 1-phosphate Signalling and Endocannabinoids System in Uterine Disorders: Endometriosis, Adenomyosis, Uterine fibroids

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Contents

Abstract

Endometriosis is a chronic inflammatory disease characterized by pelvic pain and infertility, defined by the presence of endometrium-like tissue outside the uterus. Its main pathological features include persistent inflammation and fibrosis, driven by a complex network of growth factors, cytokines, and chemokines that create a microenvironment favourable to fibrogenesis. Fibrosis is a hallmark feature, characterized by excessive extracellular matrix deposition that contributes to the tumors' rigidity and associated symptoms. Due to the lack of curative treatments and incomplete understanding of its molecular mechanisms, continued research into the pathogenesis of endometriosis remains essential.

Adenomyosis is another prevalent uterine disorder associated with heavy menstrual bleeding, pelvic pain, and infertility. It occurs when endometrial epithelial cells and stromal fibroblasts invade the myometrium, causing hypertrophy and hyperplasia of adjacent smooth muscle cells. The characteristic symptoms of adenomyosis are thought to arise from chronic inflammation, abnormal angiogenesis and neurogenesis, and altered uterine contractility. Advancing knowledge of the molecular and cellular mechanisms underlying adenomyosis could enable the development of more effective, targeted therapeutic strategies.

Uterine fibroids (leiomyomas) are the most common benign pelvic tumors in women of reproductive age. Although often asymptomatic, they can lead to abnormal uterine bleeding, pelvic discomfort, and infertility. Their pathogenesis remains complex and not fully understood.

In recent years, alterations in the sphingosine 1-phosphate (S1P) signaling pathway have been identified in several gynaecological disorders, including endometriosis, adenomyosis, and uterine fibroids. S1P is a pleiotropic bioactive sphingolipid that plays an important role in numerous biological processes, such as, proliferation, fibrosis and inflammation. S1P is synthetised intracellularly by two isoforms of sphingosine kinase (SK1 and SK2), it can be dephosphorylated by S1P phosphatases (SPP1 and SPP2), or it can be degraded by S1P lyase (SPL). After the export of S1P in the extracellular environment through non-specific or specific transporters (e.g. Spns2), several actions are mediated by its binding to five specific receptors, defined $S1P_{1-5}$. Studies have shown that endocannabinoid system (ECS) plays and important role in the establishment and maintenance of endometriosis, being involved in migration, proliferation and survival of endometriotic cells and playing an important role in the accompanying symptoms such as pain and inflammation. The ECS consists of endocannabinoid receptors including CB1, CB2, GPR18 and the transient receptor protein

channel TRPV1, the well-known endocannabinoids 2-arachidonoyil glycerol (2-AG) and anandamide (AEA) and the enzymes responsible for their metabolism.

In this work, we explored the role of S1P in endometriosis, endometriosis associated fibrosis and uterine fibroids, focusing on the fibrotic and inflammatory traits of these diseases. Additionally, we have examined endocannabinoids receptors expression in endometriotic lesion and adenomyosis tissue. Next, we investigated the cross-talk between S1P signaling axis and ECS in endometriotic epithelial cells targeting 2-AG-induced inflammation mechanism. Morely, S1P role in cell proliferation and fibrogenesis in uterine fibroids and endometriosis associated fibrosis was evaluated. Through this study we have confirmed the mRNA and protein expression of CB1, CB2, GPR18 in adenomyosis tissue and endometriosis lesion and compare it to the control group. Later experimental analysis on endometriotic epithelial cells revealed that treating this cell model with 2-AG leads to increased expression of inflammatory markers (IL-1β, IL-6, IL-8 and COX2), while the treatment with methanandamide (MAEA), the nonhydrolyzable AEA analogue, does not show the same effect. Interestingly, after interfering with $S1P_3$ the proinflammatory effect was abolished, bolding the key role of this receptor in the proinflammatory effect of 2-AG. We subsequently demonstrated that S1P significantly increased the proliferation of leiomyoma cells but not that of the healthy counter part of myometrial cells and that the mitogenic effect elicited by S1P was abolished in the presence $S1P_2$ antagonist, demonstrating that the sphingolipid stimulates cellular proliferation via $S1P_2$ signaling. Next, we clearly showed that S1P significantly activated both cytoskeleton-related proteins ezrin–radixin–moesin (ERM) and extracellular signal-regulated kinases 1/2 (ERK1/2), demonstrating a crucial role of both signaling pathways in mediating the mitogenic effect of the sphingolipid. Finally, we have stated that $S1P_3$ protein levels were markedly increased in the glandular regions of endometriotic tissues compared to control endometrium and showed a positive correlation with the degree of fibrosis. Moreover, S1P treatment played a key role in promoting fibrosis in human endometriotic epithelial cells by inducing epithelial–mesenchymal transition (EMT) and enhancing the expression of fibrotic markers. Genetic analyses further revealed that $S1P_3$ mediates the profibrotic actions of S1P. Downstream of this receptor, Ezrin and ERK1/2 signaling pathways were identified as critical mediators of the S1P-induced EMT and fibrotic response.

Overall, in this work we have highlighted the importance of S1P signaling and S1PR modulators in the pathogenesis of some uterine disorders including endometriosis, adenomyosis and uterine fibroids. Paving the way toward the new pharmaceutical approaches in the treatment of this disease.

List of abbreviations

AA	Arachidonic Acid
ABC	ATP-binding cassette
AEA	N-arachidonoylethanolamide
AMT	N-arachidonoylethanolamide membrane transporter
AFS	American Fertility Society
ASRM	American Society of Reproductive Medicine
BACE1	Beta-site APP cleaving enzyme 1
BSA	Bovine serum albumin
Cer	Ceramide
C1P	Ceramide-1-phosphate
CIB1	Calcium- and integrin-binding protein 1
Col-1αI	Collagen type 1-alfa I
COX	Cyclooxygenase
COX2	Cyclooxygenase-2
CRH	Corticotropin-releasing hormone
CTGF	Connective tissue growth factor
CYP450	Cytochrome P450
DAGL	Diacylglycerol lipase
DC	Dendritic cell
DIE	Deep endometriosis
DMEM	Dulbecco's Modified Eagle Medium
eEF1A	Eukaryotic elongation factor 1A
ECM	Extracellular matrix
ECS	Endocannabinoid System
EDG	Endothelial differentiation gene
EGF	Epithermal growth factor
EMT	Epithelial–mesenchymal transition
EndMT	Endothelial-to-mesenchymal transition
EP	Ethanolamine phosphate
ER	Estrogen receptors / Endoplasmic reticulum (depends on the context)
ERα	Estrogen receptor alfa

ERβ	Estrogen receptor beta
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellular signal-regulated kinases 1/2
ERM	Ezrin, Radixin, Moesin
F12	Nutrient mixture F12 Ham
FBS	Fetal bovine serum
FAAH	Fatty acid amide hydrolase
FFPE	Formalin-Fixed, Paraffin-Embedded
FH	Fumarate hydratase
FIGO	International Federation of Gynecology and Obstetrics
FMT	Fibroblast to-myofibroblast transdifferentiation
GPCR	G protein–coupled receptor
GnRH	Gonadotropin releasing hormone
HDACs	Histone deacetylases
HDAC-1	Histone deacetylase-1
Hex	Hexadecenal
HIF	Hypoxia-inducible factor
hTERT	Human telomerase reverse transcriptase
HUVEC	human umbilical vein endothelial cells
IFγ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
Mfsd2b	Major facilitator superfamily transporter 2b
MMPs	Matrix metalloproteinases
MMP-9	Matrix metalloproteinase-9
MSTN	Myostatin
NADA	N-arachidonoyl-dopamine

NAE	N-acylethanolamide
NAGLy	N-arachidonoyl glycine
NAPE	N-arachidonoyl phosphatidylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NGF	Nerve growth factor
NK	Natural killer cells
NO	Nitric oxide
NPS	Neuropeptide S
NSAIDs	Non-steroidal anti-inflammatory drugs
OEA	N-oleoylethanolamide
OMA	Ovarian endometrioma
PAI-1	Plasminogen activator inhibitor-1
PDCD	Pyridoxal-dependent decarboxylase conserved domain
PDGF	Platelet derived growth factor
PEA	N-palmitoylethanolamide
PHB2	Prohibitin 2
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLP	Pyridoxal 5′-phosphate
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PPARs	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome proliferator-activated receptor gamma
PR	Progesterone receptor
PTKs	Protein tyrosine kinases
qPCR	Quantitative real time PCR
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
$S1P_{1-5}$	Sphingosine 1-phosphate receptor 1-5
SDF-1	Stromal cell–derived factor 1
SKs	Sphingosine kinases
SK1	Sphingosine kinase 1

SK2	Sphingosine kinase 2
SM	Sphingomyelin
Sph	Sphingosine
Spns2	Spinster homolog 2
SPRMs	Selective progesterone receptor modulators
SPL	Sphingosine-1-phosphate lyase
SPPs	Sphingosine-1-phosphate phosphatases
SPP1	Sphingosine-1-phosphate phosphatase 1
SPP2	Sphingosine-1-phosphate phosphatase 2
SUP	Superficial peritoneal lesions
SYN	Synaptophysin
TGFβ	Transforming growth factor beta
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alfa
TNFβ	Tumor necrosis factor beta
TRAF2	Tumor necrosis factor receptor–associated factor 2
TRPV1	Transient vanilloid receptor
UAE	Uterine artery embolization
UCN	Urocortin
VEGF	Vascular endothelial growth factor
WB	Western Blot

1 . Introduction

1.1 Uterus

1.1.1 Structure and function

Uterus is a hollow, pear-shaped organ with the principal role in gestation, menstruation, and labor. The uterine cavity is seen as an inverted triangle on coronal section. placed in the female pelvis, posterior to the bladder and anterior to the rectum. The average uterine dimension in women is approximately 8 cm in length, 5 cm in width, and 4 cm in thickness, with a cavity volume ranging from 80 mL to 200 mL. The anatomic segments are divided in 4 parts and are arranged from superior to inferior: the fundus, a wide curved region where goes to the fallopian tubes; the corpus, which comprises the main uterine body; the isthmus, a narrowed segment at the lower uterine neck; and the cervix, that descends from the isthmus into the vagina (Figure 1-1) (Ameer et al. 2022).

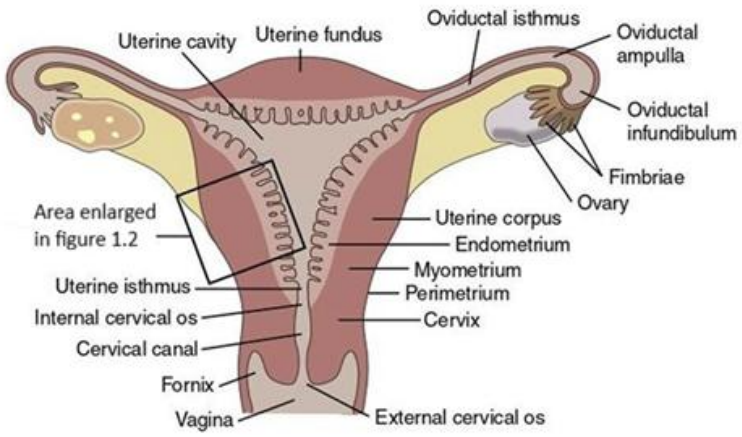

Figure 1-1 Representation of the female reproductive system (Jones and Lopez 2014)

The wall of the uterus consists of 3 distinct layers from internal to external (Figure 1-2): 1) The inner lining, endometrium, divided into a superficial functional layer consists of a lining epithelium and uterine glands, menstrual bleeding is caused from shedding of the functional layer and deeper basal layer which contains blood vessels that produce part of the menstrual

flow. Damage to the basal layer may lead to intrauterine adhesions and fibrosis. After menstruation, the basal layer gives rise to a new functional layer. In this way, the endometrium goes through hormonally controlled structural and functional changes during the menstrual cycle. 2) The middle muscular layer, myometrium, composed of smooth muscle cells layered in different orientations and contributes to uterine contractions during labor. 3) The outermost layer, the serosa or perimetrium, consists of a thin layer of epithelial cells and providing protective and friction-reducing function (Jones and Lopez 2014).

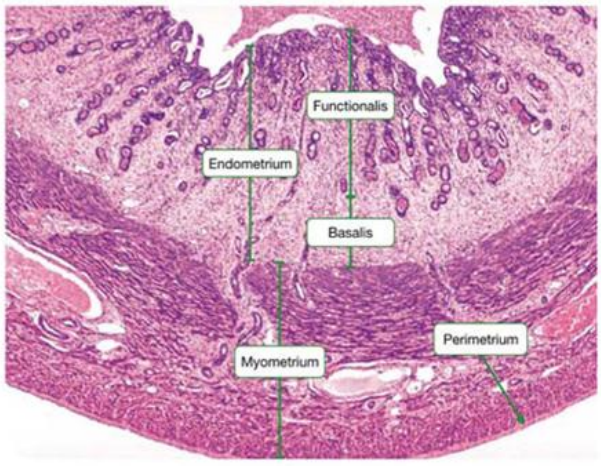

Figure 1-2. The structure of the uterine wall (Zhai et al. 2020)

1.1.2 Clinical disorders:

Uterus is involved in various gynaecologic disorders, including endometritis, endometriosis, leiomyomas, polyp, adenomyosis, endometrial hyperplasia and uterine cancer.

Endometritis refers to inflammation of the endometrium, most often linked to retained products of conception following childbirth, miscarriage, abortion or to the presence of a foreign object. The retained tissue inside the uterus can serve as a site for bacterial growth, originating from the vaginal or intestinal flora, thereby facilitating infection (Singh and Sethi 2022).

Endometriosis occurs when endometrial tissues migrate outside the uterine cavity. The most common locations are the ovaries, pelvis, and peritoneum. Once improperly implanted, endometrial cells start to grow and form a mass of endometrial tissue. Endometriosis most often affects the bilateral ovaries, and forms a blood-filled cyst, called a chocolate cyst (Zondervan et al. 2020).

Leiomyomas, also known as fibroids, are the most common benign tumors in women and often appear as multiple, well-defined masses within the uterus. While generally noncancerous, they carry a small risk of progression to malignant leiomyosarcoma. Their growth is typically stimulated during pregnancy and tends to regress after menopause. Clinically, leiomyomas may lead to abnormal uterine bleeding, recurrent miscarriages, or heavy menstrual bleeding that can cause iron-deficiency anemia in younger women (Rosa and Pidhorecky 2018).

A polyp is a well-defined overgrowth of endometrial glands and stroma, sometimes containing fibrous tissue or smooth muscle, that projects into the uterine cavity. These lesions may be asymptomatic or present with painless abnormal uterine bleeding. Small, asymptomatic polyps can regress spontaneously, while hormonal therapies such as progestins or gonadotropin-releasing hormone agonists may help reduce symptoms (Kinkel et al. 2018).

Adenomyosis is characterized by the invasion of endometrial glandular tissue into the uterine myometrium, resulting from hyperplasia of the basal endometrium. It often presents with dysmenorrhea and menorrhagia, and imaging typically reveals a uniformly enlarged, globular uterus (Paul et al. 2018).

Endometrial hyperplasia results from excessive estrogen stimulation, causing abnormal proliferation of endometrial glands. It carries an elevated risk of progression to endometrial carcinoma, necessitating close monitoring and appropriate management (Shiwani et al. 2024).

Uterine cancer is the most common gynaecologic cancer in developed countries and is strongly associated with excess estrogen. Exogenous sources of estrogen, such as tamoxifen use or unopposed estrogen replacement therapy, contribute to an increased risk. Tamoxifen acts as an estrogen antagonist in breast tissue but also stimulates estrogen receptors (ERs) in the endometrium, thereby raising the risk of endometrial cancer. Endogenous sources of estrogen include conditions such as polycystic ovary syndrome, obesity, and estrogen-secreting tumors, such as granulosa cell tumors. Endometrial cancer is a malignancy originating within the epithelial lining of the uterus. The condition has historically been classified into type 1 and type 2 endometrial cancer based on histological characteristics. Common symptoms include abnormal uterine bleeding, pelvic pain, and uterine enlargement.

1.2 Endometriosis

1.2.1 Generalities

Endometriosis is the surgical detection of endometrial tissue outside of the uterine cavity with the most common clinical symptoms as pelvic pain, dysmenorrhoea, and infertility. It is strongly connected to persistent episodes of cycling steroid hormones (Bulun et al. 2019). Endometriosis affects 5–10% of women of reproductive age. It is observed in 50–80% of women with pelvic pain and up to 50% of women with infertility. Despite this high prevalence, diagnosis time ranges from 4 to 11 years, with 65% of women being initially misdiagnosed (Taylor et al. 2021). Endometriosis is typically classified according to revised criteria formulated by the American Fertility Society (AFS) and American Society of Reproductive Medicine (ASRM), including lesion size, location and extent of adhesions, into four stages from 'minimal' to 'severe' according to the extent of disease observe (American Society For Reproductive Medicine 1997). However, no correlation exists between the severity of symptoms and the staging system. Endometriosis is specifically defined by its histology features as the presence of extrauterine lesions consisting of endometrial glands, endometrial stroma, and/or hemosiderin-laden macrophages. As it is shown in Figure 1-3, these lesions are divided based on their location and depth and described as superficial peritoneal lesions (SUP), ovarian endometrioma (OMA), or deep endometriosis (DIE) (Agarwal et al. 2019). SUP represents the least severe form of the disease and is characterized by the presence of endometrium-like tissue on the surface of the peritoneum, with infiltration less than 5 mm. OMA is characterized by the accumulation of endometrial tissue inside the ovaries, leading to the formation of endometriotic cysts. Often causes pelvic pain and ovarian dysfunction. DIE represents the most severe phenotype and is characterized by the infiltration of endometrium-like tissue into the deeper layers of pelvic organs, such as the intestine, bladder, or uterosacral ligaments. It causes severe pain and potential organ dysfunction (Vercellini et al. 2004).

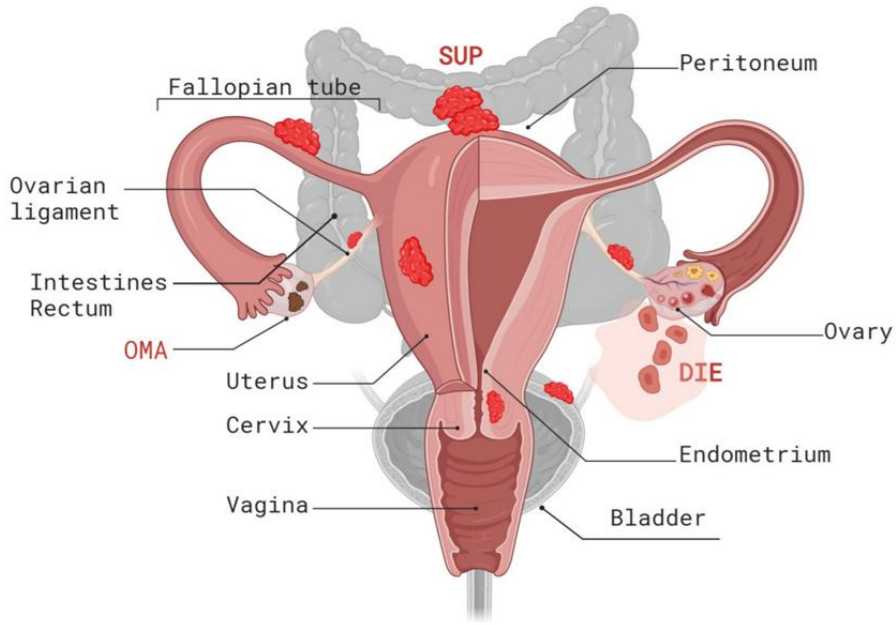

Figure 1-3. Types and locations of endometriotic lesions (Adilbayeva and Kunz 2024)

1.2.2 Pathogenesis

The origin of endometriosis remains controversial, with several models proposed to explain its initiation and the ability of endometriotic tissue to grow within the abdominal cavity and spread to extra pelvic sites (Figure 1-4) (Zondervan et al. 2020). The main hypotheses of the origins of endometrial cells at ectopic sites include retrograde menstruation, metaplasia of the coelom, vascular and lymphatic metastatic spread and neonatal uterine bleeding. However, other factors are needed to promote cell survival, proliferation and lesion formation and maintenance, including altered or impaired immunity, factors promoting angiogenesis, localized complex hormonal influences and genetic factors (Zondervan et al. 2020). The retrograde menstruation hypothesis, first proposed by Sampson in 1927, suggests that viable endometrial tissue fragments are expelled backwards through the fallopian tubes into the peritoneal cavity during menstruation (Sampson 1927). These fragments can then adhere to and invade the peritoneal lining, contributing to the development of peritoneal endometriosis. This theory is supported by epidemiological evidence linking increased menstrual exposure such as heavy bleeding, short cycles, and outflow obstruction to higher endometriosis risk (Missmer et al. 2004). The lymphatic and vascular metastasis hypothesis claims that endometrial cells and tissue fragments move from the uterine cavity through veins and lymphatic channels and colonize

distant ectopic sites. The rare occurrence of extra-pelvic endometriosis in women is supported by evidence of emboli of endometrial cells in sentinel lymph nodes is justified via this hypothesis (Jerman and Hey-Cunningham 2015). The coelomic metaplasia hypothesis, states that endometriosis can derive from the trans-differentiation of mesothelial cells, originated from the coelomic epithelium, into endometrial-like cells. Recent studies propose that multipotent mesenchymal stem cells, from the bone marrow or the endometrial niche, may differentiate into ectopic endometrial tissue. This mechanism is thought to explain unusual or deep cases of endometriosis, such as in the rectovaginal septum or extra-pelvic sites (Zondervan et al. 2020). However, some researchers argue that metaplasia alone cannot fully explain superficial peritoneal endometriosis, due to the frequent co-occurrence of various lesion types (Somigliana 2004).

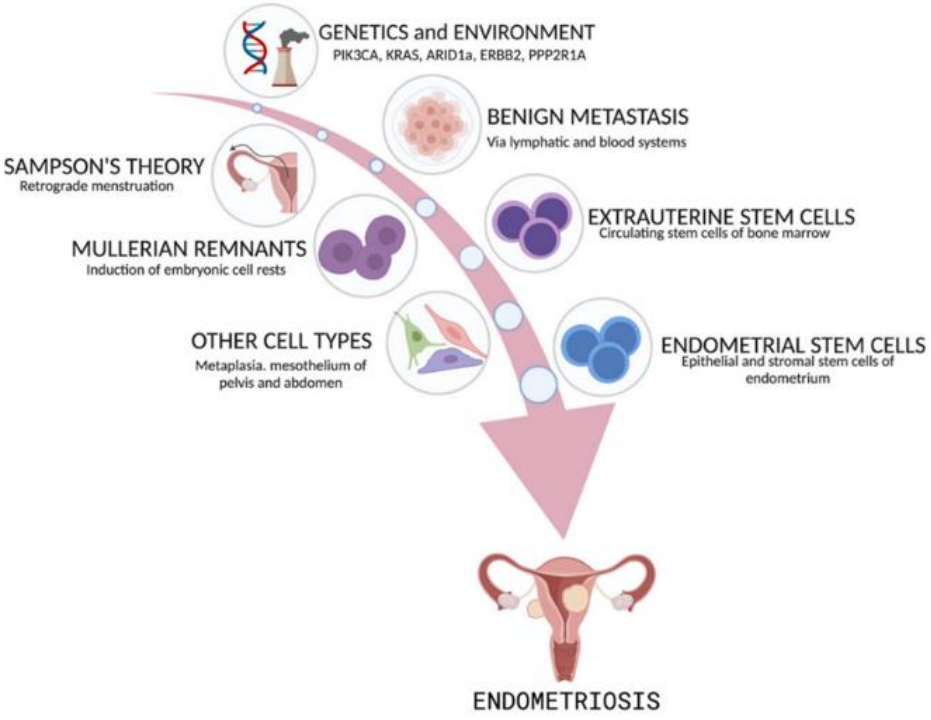

Figure 1-4. Theories regarding the pathogenesis of endometriosis (Adilbayeva and Kunz 2024)

Endometriotic lesions develop within a highly dynamic and multifaceted microenvironment shaped by inflammatory, angiogenic, and hormonal cues. These lesions are heterogeneous, containing epithelial, stromal, endothelial, glandular, and immune cells, and

exhibit distinct immunoinflammatory characteristics compared with normal endometrium (Ahn et al. 2016) (Figure 1-5). Transcriptomic analyses of menstrual-stage–matched tissues have shown that ectopic lesions display increased expression of genes linked to immune cell recruitment, cytokine–receptor signaling, cell adhesion and apoptosis, when compared to both eutopic endometrium from patients and healthy control samples (Symons et al. 2018). Most publications describe endometriosis as an **"**estrogen-dependent**"** disease. However, "steroid-dependent**"** will be a more comprehensive definition since other steroids and their receptors also play crucial roles in regulating cellular functions in both eutopic and ectopic endometrium (Saunders and Horne 2021). ERs activity drives proliferation, adhesion, and angiogenesis of ectopic lesions and are necessary for lesion establishment. It was observed that the expression level of ERα and ERβ was altered in endometriotic lesion (Burns et al. 2012). Altered expression of the progesterone receptor (PR), including epigenetic modifications at its promoter, has been identified in both endometrium and lesions of women with endometriosis. These alterations align with evidence that stromal cells from affected women show reduced responsiveness to progesterone. In parallel, increased expression of ERβ has been consistently reported in endometriosis lesions and lesion-derived stromal cells (Bulun et al. 2019). The development of ERβ-selective agonists led to their testing in endometriosis models showing encouraging preclinical results. Some data highlights the complexity of treatment for a disease characterized by intricate cell–cell interactions. For instance, while ERβ agonists may suppress epithelial cell proliferation, they also stimulate angiogenesis and the release of regulatory factors by endothelial cells derived from endometrial tissue and may influence immune cell functions such as those of macrophages (Greaves et al. 2015).

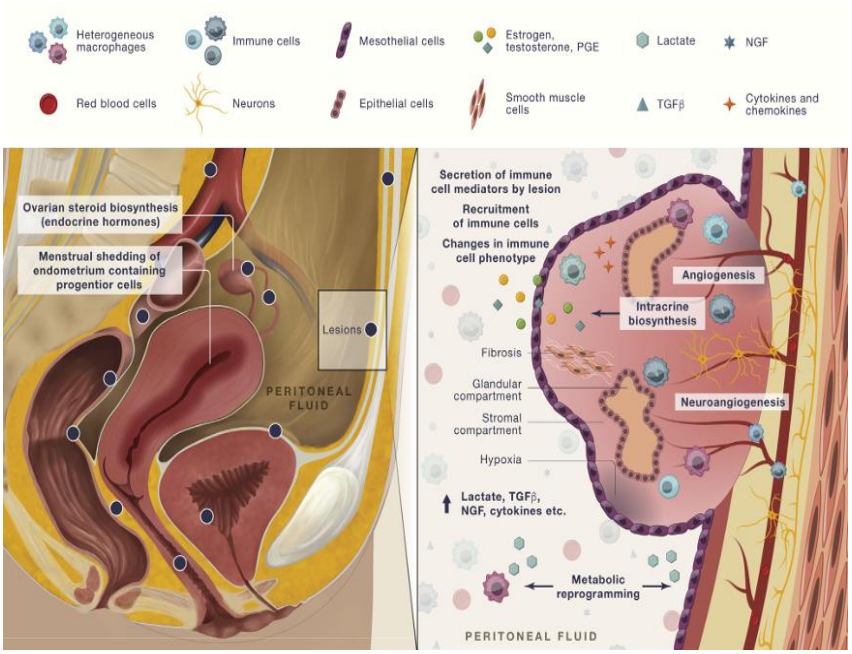

Figure 1-5. Endometriosis lesions are composed of a diverse array of cell types, creating a distinct and specialized microenvironment. (Saunders and Horne 2021)

Iron metabolism is closely linked to oxidative stress in endometriosis. Repeated bleeding within or around the ovary contributes to excess iron accumulation, leading to sustained oxidative stress. This, in turn, reduces follicle-stimulating hormone receptor expression and compromises fertilization capacity (Hayashi et al. 2020). Iron overload also promotes the generation of reactive oxygen species (ROS), which activate NF-κB in peritoneal macrophages, thereby increasing pro-inflammatory cytokines and angiogenic mediators, both of which are elevated in endometriosis compared with healthy individuals (Samimi et al. 2019). Moreover, iron overload impairs mitochondrial function, causing ATP depletion, elevated ROS production, and mitochondrial membrane potential hyperpolarization (S. Li et al. 2021). In advanced stages of endometriosis, transferrin deficiency together with iron overload significantly impairs oocyte maturation, which may explain the contribution of dysregulated iron metabolism to infertility associated with the disease (Li et al. 2020).

A fibrotic phenotype, which varies according to lesion localization, also plays a major role in persistent pain and infertility (Garcia Garcia et al. 2023). Fibrosis defined by the excessive deposition of extracellular matrix (ECM) components, results in permanent scarring, architectural disruption and loss of tissue function. Myofibroblasts are the main cells driving scar tissue remodelling and maturation; they arise either from activated fibroblasts (fibroblast-to-myofibroblast trans differentiation (FMT)) or via trans differentiation processes such as

epithelial–mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndMT). EMT is considered as a potential contributor to endometriosis in connection with stress response, invasiveness and stemness of endometriotic cells. The accumulation of fibrotic tissue within and around ovarian and peritoneal endometriotic lesions contributes to pelvic pain through adhesions, scarring and anatomical distortions (Vigano et al. 2018; Garcia Garcia et al. 2023). Nevertheless, the molecular mechanisms underlying fibrosis development in endometriotic lesions remain incompletely understood. Epithelial cells from different types of endometriotic lesions showed significant differences in the expression levels of cytokeratin, E-cadherin, and vimentin, which suggests that the EMT also plays a role in the progression of endometriosis (Albertsen and Ward 2017). In endometriosis, the overexpression of transforming growth factor- β (TGFβ) may stimulate the growth and proliferation of refluxed endometrial tissue, thereby contributing to the development of the disease (Lopez et al. 2020). Fibrosis is a hallmark of OMA, with the cyst wall frequently showing scar tissue (Anaf 2000) and is strongly associated with cortical fibrosis and stromal loss (Garcia Garcia et al. 2023). EndMT is particularly prominent in OMA, likely due to its higher angiogenic activity, while activated platelets further promote fibrogenesis by releasing profibrotic mediators such as TGFβ1, platelet derived growth factor (PDGF), epithermal growth factor (EGF) (Yan et al. 2020). TGFβ plays a central role, promoting ECM dysregulation and fibrosis through the TGFβ1/Smad pathway (Hull et al. 2012; Shi et al. 2017). Activin A, another TGFβ family member produced by macrophages, which is strongly expressed in OMA, enhances cell invasion and drives myofibroblast differentiation via STAT3-dependent Smad signaling. Interestingly, anti-Activin A antibodies reduced fibrosis in the mouse models of endometriosis (Rocha et al. 2011; Ferreira et al. 2008; Zhang et al. 2019). Studies has shown that endometriotic mesenchymal stem cells promote fibrosis through paracrine TGFβ1/Wnt1 signaling and Wnt/β-catenin activation (Li et al. 2016). The bioactive sphingolipid sphingosine-1-phosphate (S1P) also induces cytokine release, and fibrosis, with S1P enhancing fibrotic activity both independently and in synergy with TGFβ1 (Bernacchioni, Capezzuoli, et al. 2021). Plasminogen activator inhibitor-1 (PAI-1), elevated in OMA fluid (Boss et al. 2002), contributes to collagen accumulation and impaired ECM remodelling (Ghosh and Vaughan 2012). Interestingly, older cysts with repeated bleeding show higher fibrotic content, α smooth muscle actin (αSMA) expression, and iron deposition, supporting fibrosis as a progressive feature of OMA (Ding et al. 2020; Guo et al. 2015).

DIE is strongly associated with pain and infertility, largely due to smooth muscle proliferation and fibrosis (Tosti et al. 2015). Histologically, DIE implants display variable patterns, including well-differentiated glands, stromal-only, mixed differentiated, and undifferentiated cell types. The presence of epithelial and stromal endometrial cells at ectopic sites (e.g., rectum, vagina, peritoneum) reflects their ability to survive and proliferate in altered microenvironments. Recurrent tissue injury and repair characterize DIE lesions, driven by TGFβ1, vascular endothelial growth factors (VEGF), Interlukin-6 (IL-6), and Interlukin-8 (IL-8) secreted by platelets and macrophages, alongside lesion-derived PAI-1 (Figure1-6). Altered expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), in concert with local nerve fibers, contributes to the main fibrosis-related processes , including EMT, FMT and smooth muscle metaplasia (Tosti et al. 2015; Viganò et al. 2020). Several molecular and cellular pathways drive fibrogenesis in DIE. Myostatin (MSTN), a TGFβ family member, is highly expressed in endometriotic lesions, with significantly higher mRNA levels of MSTN and its receptors (ALK5, ActRIIB) in DIE compared to OMA and control endometrial tissues (Carrarelli, Funghi, et al. 2017). Through Smad2/3 activation, MSTN promotes fibroblast proliferation, myofibroblast differentiation, and ECM protein expression, contributing to fibrosis. Importantly, MSTN and TGFβ1 establish a positive feedback loop that mutually enhances their expression (Zhu et al. 2007). Activation of the Wnt/β-catenin pathway enhances stromal proliferation, migration, and collagen contraction, upregulating fibrotic markers such as connective tissue growth factor (CTGF), collagen type 1-α I (Col-1αI), αSMA, fibronectin. FOXP1, a transcription factor that promotes β-catenin acetylation, is upregulated in DIE stromal cells and inhibition of this pathway reduces fibrosis *in vitro* (Vissers et al. 2024). Aberrant activation of survival pathways also supports lesion persistence: integrins (α1β1, α2β1) engage PI3K/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling, with increased AKT and ERK phosphorylation in endometriotic stromal cells, favoring survival in fibrotic environments (Gullberg et al. 1992; Matsuzaki and Darcha 2015). Moreover, DIE lesions display tumour-like features. In fact, mutations in cancer driver genes (*TP53*, *PTEN*, *ARID1A*, *PIK3CA*, *KRAS*, *PPP2R1A*) have been identified (Guo 2018). These mutations influence fibrogenesis; e.g., *TP53* loss enhances fibroblast activation and ECM deposition via TGFβ1 upregulation (Guo 2018). Their precise functional role remains unclear but may contribute to invasive and fibrotic characteristics.

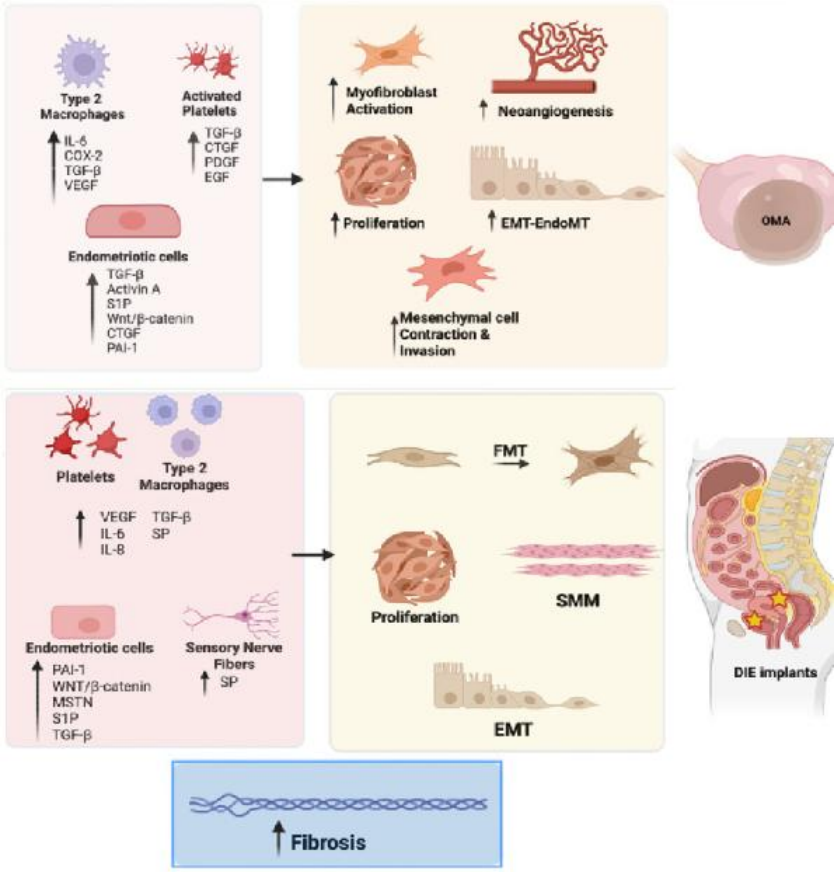

Figure 1-6. Principal cellular types and molecules involved in the development of DIE and OMA-related fibrosis (Garcia Garcia et al. 2023)

Based on various evidence endometriosis is classified as an inflammatory disorder, including reports of the altered peritoneal environment in women with endometriosis (Figure 1-7). Endometriosis lesions recruit a large number of immune cells and abnormal production of pro-inflammatory regulatory cytokines (Saunders and Horne 2021). Endometriosis lesions are often attached to the sites where they are exposed to peritoneal fluid containing immune cells, cytokines and growth factors for instance, the peritoneal wall or ovaries. Pro-inflammatory factors increased in the peritoneal fluid of women with endometriosis include interleukins (IL-1, IL-6, IL-8 and IL-33), tumor necrosis factor (TNF), and insulin-like growth factor 1(IGF-1) (Forster et al. 2019; Cheong 2002). In endometriosis, IL-1β promotes the proliferation of ectopic endometrial cells and induces the production of IL-6 and IL-8. Notably, IL-8 plays a critical role in lesion development by enhancing cell proliferation and apoptosis. Oxidative stress also contributes, as ROS-derived 4-hydroxy-2-nonenal triggers IL-8 release from monocytes, linking redox imbalance to endometriosis pathophysiology (Thézénas et al. 2020). Ectopic endometrial cells themselves produce IL-6, which influences interferon-γ (IFγ)

activity and increases ICAM-1 expression in macrophages of affected patients (Samimi et al. 2019). IL-6 can further regulate Notch1 expression, thereby promoting fibrosis, angiogenesis, and disease progression (Yong Song et al. 2020). Neutrophil infiltration has been elevated in the peritoneal fluid of endometriosis patients compared to that of disease-free women (Milewski et al. 2011). This could be a result of increased concentrations of neutrophil chemo-attractants such as IL-8 present in endometriosis patient plasma and peritoneal fluid (Milewski et al. 2011). Macrophages and mast cells have been shown to play an important role at the sites of inflammation through release of chemokines and subsequently neutrophil recruitment. Peritoneal macrophages isolated from endometriosis patients also demonstrate increased protein expression of the proinflammatory cytokines TNFα, IL-6, and IL-1β (Montagna et al. 2008). In the peritoneal fluid of women with endometriosis IL-6 and TGFβ1 have been shown to reduce natural killer (NK) cells cytolytic activity. Particularly, IL-6 downregulated NK cell cytolytic granule components (Kang et al. 2014). In addition, the cell-mediated regulated by T lymphocytes (T cells) and humoral components of adaptive immunity that are regulated by antibodies produced by B lymphocytes (B cells) have also been implicated in endometriosis pathophysiology (Symons et al. 2018). Peroxisome proliferator-activated receptor (PPAR), a ligand-activated transcription factor, has been shown to reduce monocyte migration stimulated by peritoneal fluid from endometriosis patients *in vitro*, highlighting its involvement in the disease's pathophysiology. Furthermore, activation of PPAR with rosiglitazone has been reported to induce regression of endometriotic lesions (Aytan et al. 2007). Collectively, these cytokines, mainly secreted by activated peritoneal macrophages, drive ectopic endometrial adhesion, proliferation, and growth.

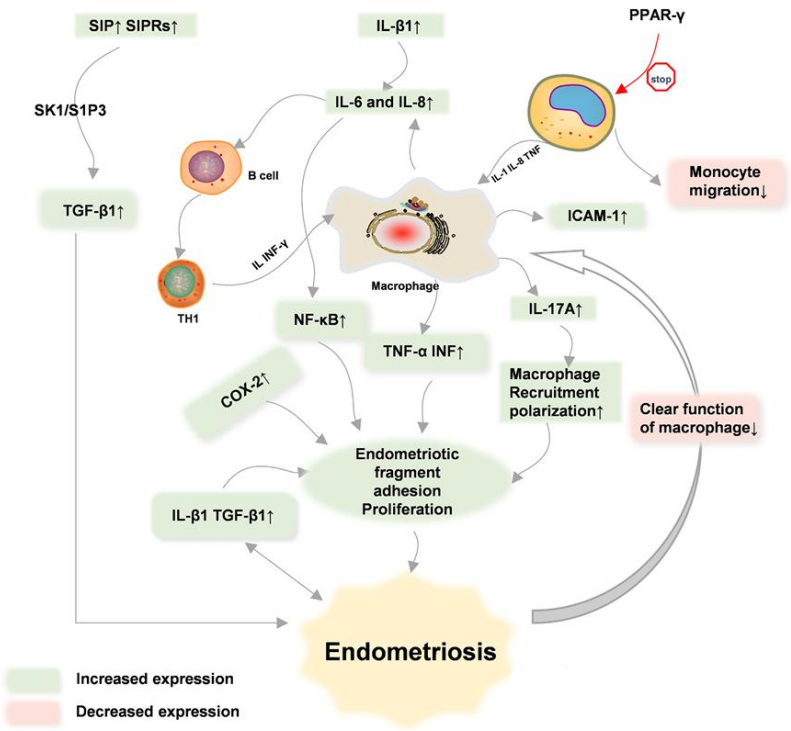

Figure 1-7. Cytokines metabolism hallmarks in endometriosis (Lu et al. 2023)

Endometriotic lesions must establish a new blood supply to support survival and growth therefore angiogenesis, regulated by immune cells and related mechanisms, is integrated to the pathogenesis of endometriosis. A hallmark of endometriotic lesions is their rich vascularization, which has prompted the hypothesis that inhibiting new blood vessel formation (antiangiogenic therapy) could represent an effective treatment strategy for endometriosis. Surgical removal of endometriotic lesions has been shown to decrease serum VEGF in patients, suggesting that lesion presence in the peritoneum has a profound effect on the microenvironment. In human endometriotic lesions, VEGF expression has been localized to the stroma, glandular epithelium (Ceyhan et al. 2008). Furthermore, in animal models analogous immune cell infiltration to endometriotic lesions and angiogenic activity have been observed (Lin et al. 2006). In mice lesion weight and vascularity was decreased after depletion of macrophages using chlodronate liposomes, monoclonal antibodies, or ganciclovir (Capobianco et al. 2011; Bacci et al. 2009). Similarly, cabergoline treatment which is used to reduced infiltration of macrophages and mast cells in a mouse model led to decreased number of immature vessels in lesions (Novella-Maestre et al. 2012). These states clearly suggest that immune cells generate a pro-angiogenic peritoneal microenvironment in endometriosis. In

addition, various chemokines including stromal cell-derived factor-1 (SDF-1) can have pro-migratory effects on endothelial cells. SDF-1 is a constitutively expressed and inducible chemokine that regulates multiple physiological processes and has also been implicated in endometriosis pathophysiology (Virani et al. 2013). Some studies have demonstrated increased expression of hypoxia-inducible factor (HIF)-1a and SDF-1 in endometriotic lesions as well as increased SDF-1 in the systemic circulation of endometriosis patients. In fact, interfering with the SDF-1/CXCR4 axis led to significant reduction of endothelial progenitor cells in mouse endometriotic lesions and reduced lesion size and vascularization in mice (Virani et al. 2013; Becker et al. 2008). Women with endometriosis are more likely to have coexisting gynecologic conditions than women without endometriosis. A strong correlation has been shown between endometriosis and adenomyosis and uterine fibroids, as well as associations with endometrial cancer. These pathogeneses can be influenced by diagnostic biases and failure to distinguish between diagnoses in women undergoing hysterectomy and those in women with intact uteri. In addition, a meta-analysis showed an increased risk of several autoimmune diseases among women with endometriosis, but most of the studies were of insufficient quality. In addition, increased risks of melanoma, non-Hodgkin's lymphoma, and thyroid and endometrial cancers has been observed in some cases but with less consistent evidence (Zondervan et al. 2020).

1.2.3 Treatment

Current therapies for endometriosis primarily aim to control symptoms and slow lesion progression, but they do not offer a definitive cure (Brichant et al. 2021). The goal is to stop cyclic menstruation: by blocking ovarian estrogen secretion or by causing a pseudopregnancy state (Vannuccini et al. 2022). First-line treatment typically combines non-steroidal anti-inflammatory drugs (NSAIDs), which reduce prostaglandin production and inflammation through cyclooxygenase (COX) enzymes inhibition, with progestins such as medroxyprogesterone, norethisterone or dienogest. These progestins exert beneficial effects by limiting neovascularization, inflammation, cell proliferation and neurogenesis (Buggio et al. 2017). When first-line therapies are insufficient, gonadotropin releasing hormone (GnRH) agonists or antagonists are used to suppress estrogen to postmenopausal levels (Vannuccini et al. 2022). However, treatment effectiveness is often compromised by progesterone resistance and burdensome side effects. Surgical approaches, including laparoscopy, can provide

temporary relief from pain and infertility but are not curative, as recurrence occurs in up to 50% of patients within two years (Hamdan et al. 2015). Patients with endometriosis currently lack treatments that control both pain and lesion growth while preserving fertility. Management typically requires a multidisciplinary approach, combining NSAIDs, hormonal therapy, and/or surgical lesion removal, though hormone-based treatments can hinder conception. Key research goals include understanding the mechanisms of lesion establishment and survival to develop next-generation therapies that effectively eliminate lesions, prevent recurrence and minimize side effects (Tanaka et al. 2020). Collectively, these limitations highlight the urgent need for innovative therapeutic strategies, particularly non-hormonal options, to achieve more effective and sustainable management of endometriosis.

1.3 Adenomyosis

1.3.1 Generalities

Adenomyosis is a uterine disorder, more commonly diagnosed in women of reproductive age, characterized by the presence of ectopic endometrial glands and stroma within the myometrium, surrounded by hyperplastic smooth muscle (Figure1-8) (Farquhar and Brosens 2006). Clinically, it manifests with pelvic pain, abnormal uterine bleeding, and infertility. The most common symptoms are dysmenorrhea and dyspareunia, although presentations can vary, and in some cases, the condition may remain asymptomatic (Farquhar and Brosens 2006).

1.3.2 Pathogenesis

The pathogenic mechanisms underlying adenomyosis remain under debate; however, abnormalities in sex steroid hormones, inflammation, fibrosis, and neuroangiogenesis are considered central contributors to its defining symptoms (Zhai et al. 2020; Vannuccini et al. 2017; Carrarelli et al. 2015). Current theory suggests that adenomyosis develops via the down-growth and invagination of the endometrial basalis into the myometrium, facilitated by an altered or disrupted junctional zone (Bergeron et al. 2006). In fact, recent genetic evidence strongly supports the idea that adenomyosis arises from the basal layer of the endometrium

invading and growing into the myometrium (Bulun et al. 2021). Endometrial invasion in adenomyosis may occur either in a predisposed myometrium or at sites of trauma to the endometrial–myometrial interface. According to one theory, repeated uterine auto-traumatization initiates the recurrent tissue injury and repair mechanism, leading to chronic proliferation and inflammation. This ongoing process is proposed as a key driver in the pathophysiology of adenomyosis (Leyendecker et al. 2015). Another proposed mechanism suggests that adenomyosis, similar to endometriosis, may arise through metaplasia of ectopic intra-myometrial endometrial tissue (Hufnagel et al. 2015). More recent findings indicate that adult stem cells, once activated by tissue injury, could contribute to ectopic endometrial growth via disruption of the endometrial stem/progenitor cell niche. However, further studies are needed to clarify the role of endometrial stem/progenitor cells in the onset and progression of adenomyosis (Gargett et al. 2015).

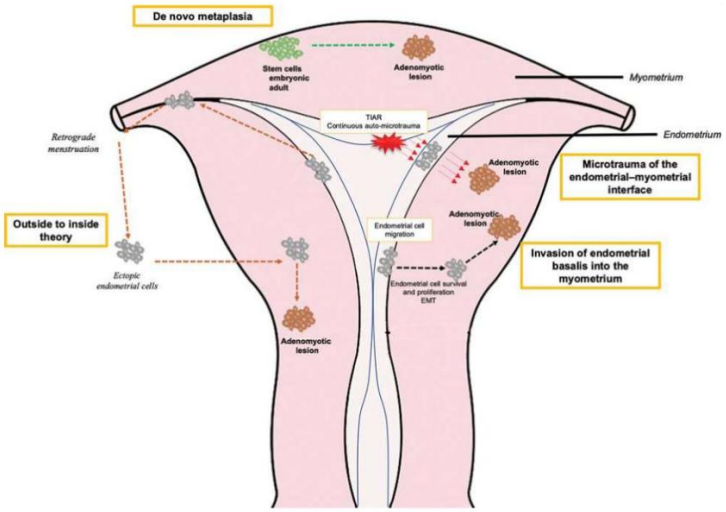

Figure 1-8. Theories on the Origin of Adenomyosis (Zhai et al. 2020)

Women with adenomyosis experience a significant imbalance in estrogen and progesterone signaling during their reproductive years (Rossi et al. 2022). Notably, adenomyotic lesions show increased local estrogen production despite normal circulating estradiol levels, a phenomenon attributed to elevated aromatase expression (Kitawaki 2006). *ERα* gene polymorphisms and increased *ERβ* expression in the myometrium contribute to hyperplasia and are associated to an enhanced incidence of adenomyosis. Reduced activity of

progesterone receptors (PR), particularly decreased PR-B expression, diminishes progesterone effects and promotes abnormal endometrial growth (Mehasseb et al. 2011). The early development of adenomyosis requires endometrial tissue to invade the myometrium. In fact, EMT appears to be a critical process that enhances the migratory and invasive potential of adenomyotic lesions. Primarily, EMT was considered because the downregulation of E-cadherin in association with upregulation of vimentin in the epithelial cells of adenomyotic lesions was reported. Later, other studies confirmed that EMT can be triggered by ER expression, reduced PR levels, platelet activation, and chronic hyperperistaltic activity (Rossi et al. 2022; Chen et al. 2010). Fibrosis is a key mechanism in adenomyosis pathogenesis and can be triggered by multiple factors. TGFβ signaling regulates metaplasia and smooth muscle fibrosis via a Smad2/3-dependent pathway (Cheong et al. 2019). Myostatin and activin A, which promote myometrial growth, are also upregulated in the eutopic endometrium of adenomyosis patients (Carrarelli et al. 2015).

Adenomyosis is characterized by abnormal vascularization and neuroangiogenesis, which drive lesion progression, heavy menstrual bleeding and pain. Increased microvessel density in ectopic and eutopic endometrium is driven by VEGF overexpression, induced by hypoxia and HIF-1, and further promoted by TGFβ family members such as follistatin and activin A (Filippi et al. 2016; Bulun et al. 2021; Carrarelli, Yen, et al. 2017). Hypoxia, often caused by junctional zone injury and impaired perfusion, contributes to disease progression. Neuroangiogenesis amplifies pain through elevated neurogenic factors, including nerve growth factor (NGF), synaptophysin (SYN), and MAP2, which stimulate mast cell activation and the production of inflammatory mediators and peripheral nociceptors (Zhai et al. 2020). Inflammatory cytokines such as IL-1 and TNFβ further enhance NGF levels, linking inflammatory and neurogenic pathways in adenomyosis (Luddi et al. 2019).

Inflammation plays a central role in the pathogenesis of adenomyosis. Elevated levels of IL-1β, corticotropin-releasing hormone (CRH), and urocortin (UCN) have been detected in adenomyotic nodules, since CRH/UCN can activate cyclooxygenase-2 (COX2), this may increase prostaglandin synthesis and pain (Carrarelli, Funghi, et al. 2017). The tissue injury–repair process involves IL-1–induced COX2 activation, leading to excess prostaglandin E2 (PGE2) production (Leyendecker et al. 2015). Hyperestrogenism further contributes by stimulating IL-10, an immunosuppressive cytokine, whose high expression in eutopic and ectopic endometrium may allow ectopic foci to evade immune clearance (Wang et al. 2009). Additionally, activation of the toll like receptor 4 (TLR4) pathway in stromal cells promotes

cytokine and growth factor release, immune cell recruitment, and enhanced proliferation and invasion of endometrial cells. This amplifies local inflammation and drives adenomyosis progression (Guo et al. 2016). Overall, an imbalance between pro- and anti-inflammatory signals, combined with platelet activation, drives fibrosis and inflammation in adenomyosis (Figure 1-9).

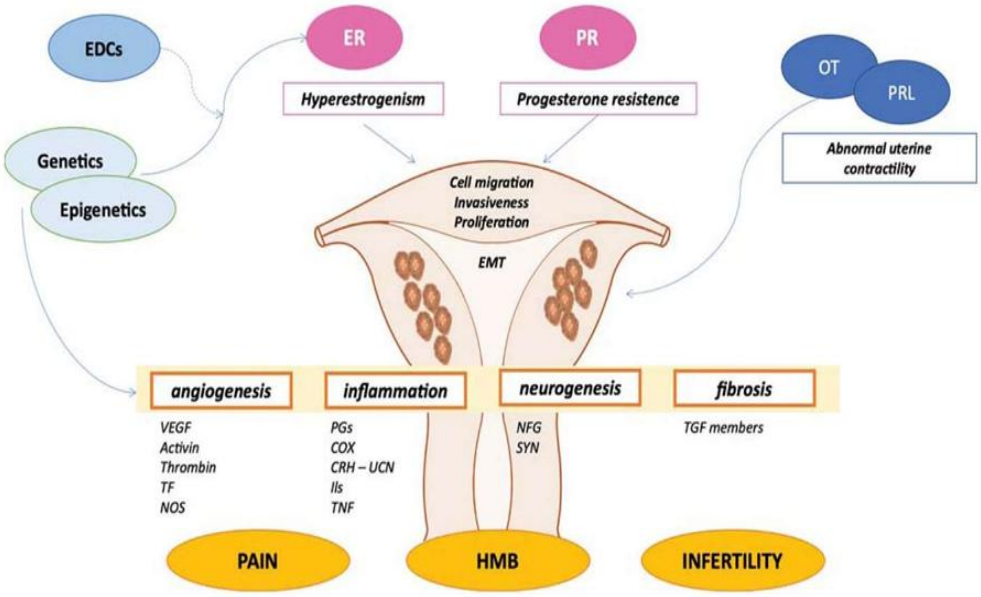

Figure 1-9. Pathogenesis of adenomyosis (Zhai et al. 2020)

Noteworthy, adenomyosis and endometriosis share several features and often coexist in certain patient subgroups. Endometriosis is observed in over 90% of patients presenting adenomyosis with a difference in the prevalence of endometriosis according to the site of the adenomyosis lesion in the myometrium (Chapron et al. 2017). Although now are considered distinct conditions due to differences in pathogenesis, risk factors, and clinical presentation they still exhibit significant similarities in definition, symptoms, and molecular alterations (Li et al. 2014).

1.3.3 Treatment

Adenomyosis is a uterine disorder affecting women of various ages and presenting with a range of symptoms. Management strategies remain debated, and only a limited number of clinical studies have investigated medical or surgical treatments for this condition. Vannuccini and colleagues (Vannuccini et al. 2018) note that, to date, no drug is specifically approved for adenomyosis, and no formal guidelines exist for its optimal management. Consequently, medical therapies are chosen based on the underlying pathogenic mechanisms of the disease (Vannuccini et al. 2018). Today, patients with adenomyosis suffering from dysmenorrhea are given NSAIDs which are effective in alleviating pain by inhibiting prostaglandin synthesis. NSAIDs and other analgesics remain the first-line treatment for women with adenomyosis who wish to maintain fertility (Gruber and Mechsner 2021). Although evidence on the efficacy of estrogen–progestin contraceptives specifically for adenomyosis is limited, these agents are effective as first-line treatments for heavy menstrual bleeding and dysmenorrhea (Wong et al. 2009). Progestins, such as Dienogest, thanks to their antiproliferative and anti-inflammatory actions, are indicated in the medical management of adenomyosis primarily to control pain symptoms (Vannuccini et al. 2018). The other in use medication are GnRH analogues, both agonists and antagonists which are key in adenomyosis management by suppressing gonadotropin release and reducing estrogen, leading to uterine shrinkage and pain relief. Selective progesterone receptor modulators (SPRMs), such as mifepristone, modulate PRs to reduce uterine size, control bleeding, and alleviate pain. Mifepristone also promotes apoptosis in endometrial cells and is suitable for long-term therapy due to its low cost and favourable safety profile (Selntigia et al. 2024). The levonorgestrel-releasing intrauterine device (LNG-IUD) has proven highly effective for long-term management of adenomyosis, reducing abnormal uterine bleeding and decreasing uterine volume (Vannuccini et al. 2018). Uterine artery embolization (UAE) induces ischemic necrosis of adenomyotic lesions by blocking uterine blood supply, effectively reducing symptoms while sparing surrounding tissues. It is suitable for women with symptomatic adenomyosis who have completed childbearing but not recommended for those planning future pregnancies due to potential adverse reproductive outcomes (Zhou et al. 2016). On the other hand, uterus-sparing surgical approaches aim to remove adenomyotic tissue, reduce uterine size, and relieve symptoms. They are particularly considered for women wishing to preserve fertility or when medical therapies are ineffective or contraindicated (Pecorella et al. 2024).

1.4 Uterine fibroids

1.4.1 Generalities

Uterine fibroids, also referred to as leiomyomas or myomas, are benign pelvic tumors of the myometrium and represent the most common neoplasms in women of reproductive age. Despite being benign, fibroids represent a major source of morbidity. They can cause heavy or prolonged menstrual bleeding, often leading to iron deficiency and anaemia, as well as urinary (e.g., frequency, nocturia, retention) and gastrointestinal symptoms (e.g., diarrhea, constipation). Fibroids are also associated with infertility and adverse obstetric outcomes. Nonetheless, up to 70% of women remain asymptomatic, even with large fibroids (Pavone et al. 2018). Fibroids consist of smooth muscle cells and fibroblasts embedded within an abundant ECM which contributes to their rigid structure and is a key factor underlying abnormal uterine bleeding and pelvic pain (Stewart 2001; Bulun 2013). Owing to the hormonal dependence, fibroids predominantly occur during the reproductive years, are rare before menarche, and usually regress after menopause (Marsh and Bulun 2006). The International Federation of Gynaecology and Obstetrics (FIGO) developed a classification system for identifying the causes of abnormal uterine bleeding in women of reproductive age, primarily based on imaging findings. This system employs an 8-point (Figure 1-10) numeric scale to define fibroid location in relation to the endometrium (submucosal surface) and the serosal surface, where lower scores reflect a more central position (Munro et al. 2011).

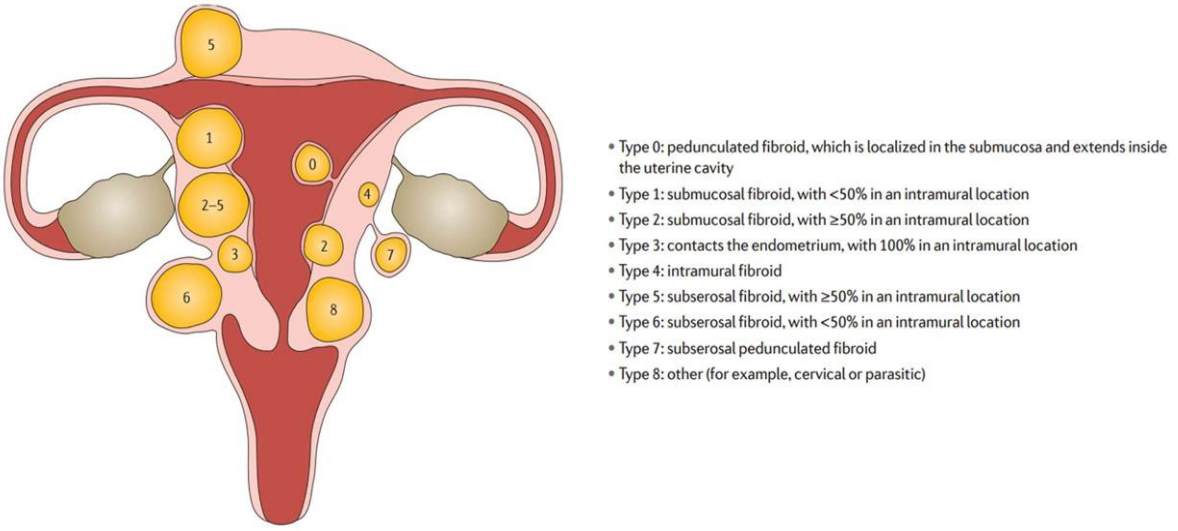

Figure 1-10. The classification of uterine fibroids by FIGO (Stewart et al. 2016)

1.4.2 Pathogenesis

Uterine fibroids emerges from the neoplastic transformation of a mutated myometrial smooth muscle stem/progenitor cell and proliferate under the influence of the ovarian steroid (Yang et al. 2022; Buyukcelebi et al. 2023). Briefly, the myometrium normally undergoes cyclical proliferation in response to ovarian hormones, but genetic mutations in these cells can trigger neoplastic transformation. The expansion of mutated cells, together with fibroblasts and excess extracellular matrix, ultimately leads to the formation of clinically detectable fibroids (Figure 1-11) (Bulun et al. 2025). Fibroid stem cell self-renewal is supported by paracrine signaling via the WNT–β-catenin pathway, which stimulates TGFβ3 expression, driving fibronectin production and cell proliferation (Bulun et al. 2015; Ono et al. 2013). Animal xenograft models further demonstrate that steroid hormones are essential for tumor growth (Ishikawa et al. 2010). Moreover, polymorphisms in ERs and PRs, as well as alterations in their signaling pathways, have been implicated in fibroid development (Hsieh et al. 2003). Enhanced local aromatase expression, which converts circulating precursors into estrogens, also contributes to fibroid growth, particularly in black women (Ishikawa et al. 2009).

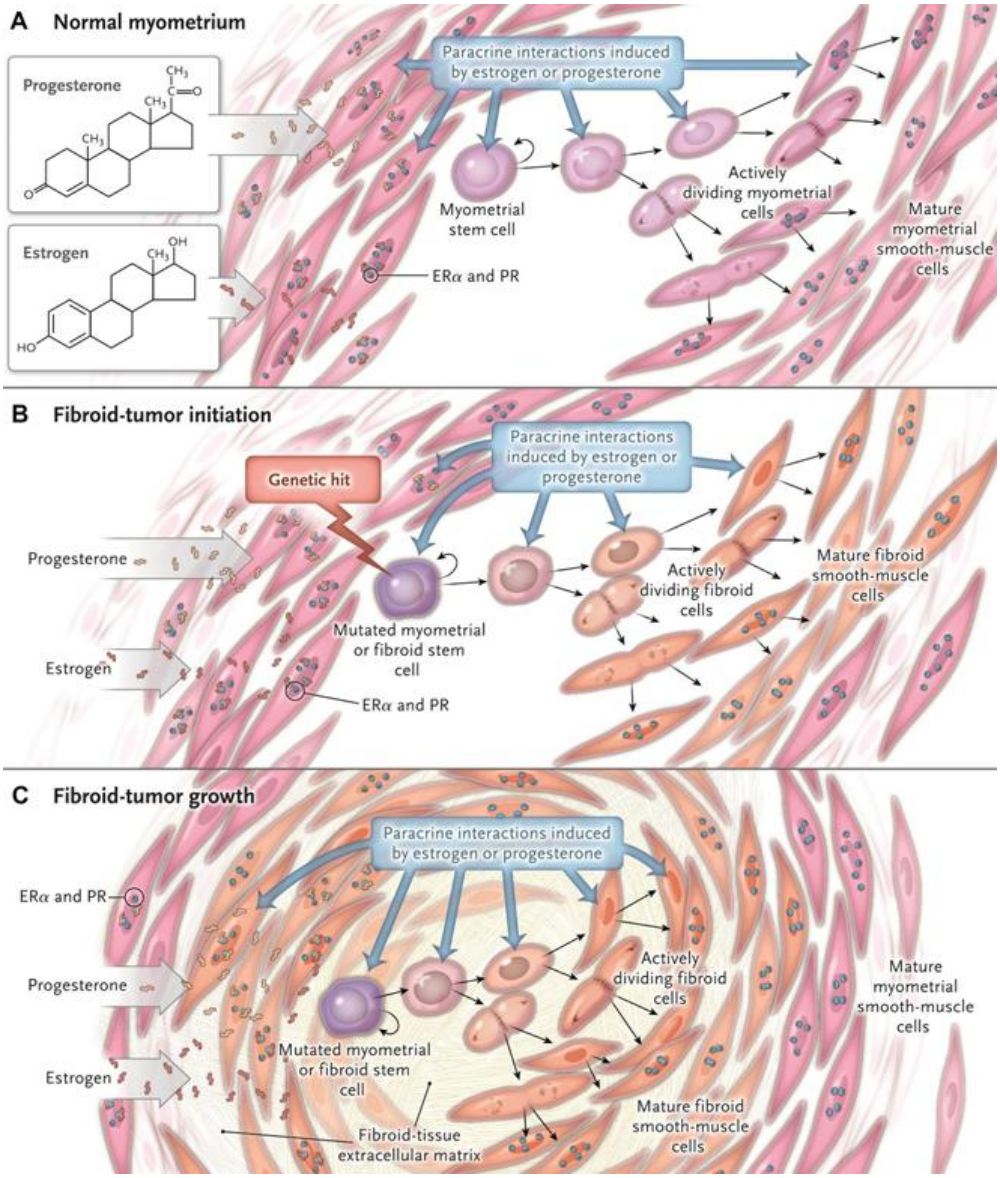

Figure 1-11. Schematic photo representing the emerging of uterine fibroids (Bulun et al. 2025)

Uterine fibroids are estrogen-dependent tumors, closely associated with reproductive age (Ishikawa et al. 2009). Indeed, dysregulation of estrogen and progesterone signaling pathways plays a central role in their pathobiology (Borahay et al. 2017). *In vitro* studies have shown that estradiol and progesterone enhance fibroid cell (Cermik et al. 2002), with estrogen and its receptor, Erα, inducing PRs expression and thereby facilitating progesterone-mediated effects. In addition, specific driver mutations play a critical role in fibroid initiation by influencing stem cell differentiation. Hierarchical gene clustering has identified four main pathogenetic subgroups of uterine fibroids, defined by somatic mutations or chromosomal alterations: the *MED12* group, the *HMGA2* group, the fumarate hydratase (*FH*) group, and a rare subgroup

associated with deletions of *COL4A5* and *COL4A6* (Mehine et al. 2013; 2014). MED12, a component of the mediator complex that regulates transcription (Borggrefe and Yue 2011), is the most frequently mutated gene. These mutations alter the interaction between MED12 and cyclin C, thereby affecting β-catenin transcriptional activity (Mehine et al. 2014; Turunen et al. 2014). Fibroids with *MED12* mutations show increased expression of *WNT4*, a β-catenin activator (Markowski et al. 2012), and *MED12* deficiency has been linked to TGFβ pathway activation, promoting drug resistance and fibroid (Huang et al. 2012). Together, these findings suggest a mechanism where *MED12* mutations, WNT–β-catenin activation, and hyperactive TGFβ signaling cooperate to drive stem-cell renewal, cell proliferation, and fibrosis. The *HMGA2* subgroup is characterized by dysregulation of HMGA2, a transcription-regulating factor (Hodge et al. 2009). This may promote fibroid growth by upregulating *CDKN2A*, which encodes *ARF (p14)*, a protein that normally preserves senescence (Markowski et al. 2011). Importantly, fibroids lack Let-7 miRNA, a negative regulator of HMGA2, thereby sustaining HMGA2 activity and bypassing senescence (Peng et al. 2008). In the FH subgroup, inactivating mutations in the fumarate hydratase enzyme, essential for the Krebs cycle, reprogram cellular metabolism and activate hypoxia signaling (Ono et al. 2013). Finally, deletions of *COL4A5* and *COL4A6* are associated with diffuse leiomyomatosis with Alport syndrome and, more rarely, with non-syndromic fibroids (Mehine et al. 2013; 2014).

Uterine fibroids are a fibrotic disorder characterized by exaggerated wound-healing responses, continuous ECM accumulation, and tissue rigidity (Leppert et al. 2006; Rafique et al. 2017). Fibroids contain ~50% more ECM, mainly collagens, fibronectin and versican, than adjacent myometrium (Islam et al. 2018). This excess of ECM generates abnormal mechanical stress transmitted to cells through mechanotransduction, activating downstream signaling pathways that drive fibroid pathogenesis (Rafique et al. 2017; Leppert et al. 2014). Increased ECM stiffness contributes to the rigid structure of leiomyomas and is associated with abnormal bleeding and pelvic pain. Fibrotic remodelling involves inflammatory cell recruitment and activation of fibroblasts into myofibroblasts, which synthesize collagen, remodel ECM, and contract surrounding tissues (Kisseleva and Brenner 2008; Hinz et al. 2012). Leiomyomas contain αSMA positive, desmin-negative cells, along with high collagen deposition, consistent with myofibroblast activity (Protic et al. 2016). Physiological processes such as ovulation, menstruation, and implantation, together with mechanical stress, hypoxia and oxidative stress may promote chronic uterine inflammation (Wegienka 2012; Santulli et al. 2013). Growth of fibroids is further driven by growth factors (Ciarmela et al. 2011). Among these, TGFβ plays

a central role by promoting myofibroblast differentiation and fibrosis (Fallowfield et al. 2007). Fibroids overexpress TGFβ receptors compared with normal myometrium, and its downstream effectors, such as tissue matrix metalloprotease inhibitors and plasminogen activator inhibitors, favour ECM accumulation. In fibroids, activin A enhances ECM protein expression (Islam et al. 2014) and its role in fibrogenesis is highlighted by the observation that ulipristal acetate, a fibroid therapy, reduces activin A expression *in vitro* (Ciarmela et al. 2014).

1.4.3 Treatment

Regarding to the treatment of uterine fibroids the international obstetrics and gynecology societies generally advocate a stepwise strategy, beginning with pharmacological or minimally invasive approaches before considering surgical intervention (Giuliani et al. 2020). Hormonal treatments such as combined oral contraceptives, progestins and other agents to manage heavy bleeding and other symptom (Jiang et al. 2014). GnRH agonists/antagonists used particularly when symptom relief or reduction in fibroid size is needed, sometimes prior to surgery. These can lower estrogen to shrink fibroids temporarily. Emerging treatments include SPRMs, which show promise for symptom relief and perhaps fibroid size reduction (Donnez et al. 2012). Recent advances highlight the therapeutic potential of progesterone receptor modulators, yet surgical options continue to be the most common treatment for women with symptomatic fibroids (Giuliani et al. 2020). Although recent research has expanded our understanding of fibroid pathophysiology, further studies are required to better define their genetic and phenotypic profiles, identify new therapeutic targets, ideally compatible with fertility, and improve personalized treatment.

1.5 Sphingolipids

Sphingolipids are a widespread class of lipids found in all eukaryotic cells. They were first discovered in the brain in the late 19th century by Thudicum (Hannun and Obeid 2018). Their fundamental structure consists of a sphingoid base, most commonly sphingosine (Sph), that can be N-acylated with fatty acid chains of varying lengths and saturation and/or modified with a polar head group at position one (Quinville et al. 2021; Merrill 2008). These head groups

range from simple phosphate moieties to more complex structures, such as phosphocholine in sphingomyelin (SM) or diverse glycans in glycosphingolipids (Merrill 2008). Beyond serving as essential structural components of membranes, several members of this lipid family including ceramide (Cer), ceramide-1-phosphate (C1P), Sph, and S1P are now recognized as bioactive signaling molecules. They play pivotal roles in modulating physiological and pathological processes such as cell proliferation, survival, senescence, migration, inflammation, and angiogenesis (Hannun and Obeid 2018; 2008).

1.6 Sphingosine 1-phosphate (S1P)

1.6.1 Generalities

Sphingosine 1-phosphate (S1P) was initially thought to be only a metabolic intermediate of Sph (Blaho and Hla 2014). But nowadays, S1P has been implicated in diverse physiological and pathological processes, including cell survival, proliferation, migration, cytoskeletal remodelling, calcium signaling, vascular development, inflammation, and immunity (Richard L Proia and Hla 2015; Spiegel and Milstien 2003). Cytokines and growth factors regulate S1P signaling by modulating its synthesis and degradation, while S1P receptor activation can, in turn, enhance growth factor and cytokine signaling (Lebman and Spiegel 2008). S1P functions either as an intracellular second messenger or through “inside-out” signaling, where it is exported via specific Spinster homolog 2 (Spns2) or non-specific transporters to activate its five receptors ($S1P_{1–5}$) (Figure 1-12). These receptors are part of the G protein-coupled receptor (GPCR) family and are linked to distinct heterotrimeric G protein complexes, which subsequently regulate diverse intracellular signaling pathways (Spiegel and Milstien 2011).

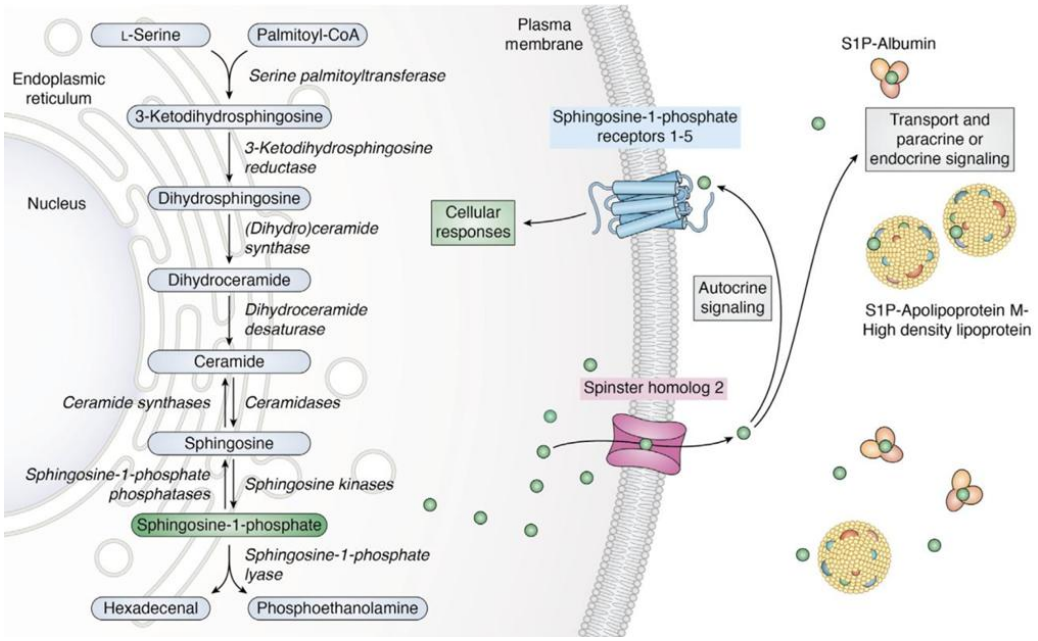

Figure 1-12. Representing S1P metabolism and transport and inside-out signaling of S1P (Weigel et al. 2023)

S1P cellular levels are tightly controlled by a balance between synthesis, mediated by sphingosine kinases (SKs), and degradation through lipid phosphate phosphatases, S1P phosphatases (SPPs), or S1P lyase (SPL) (Figure 1-13) (González-Fernández et al. 2017).

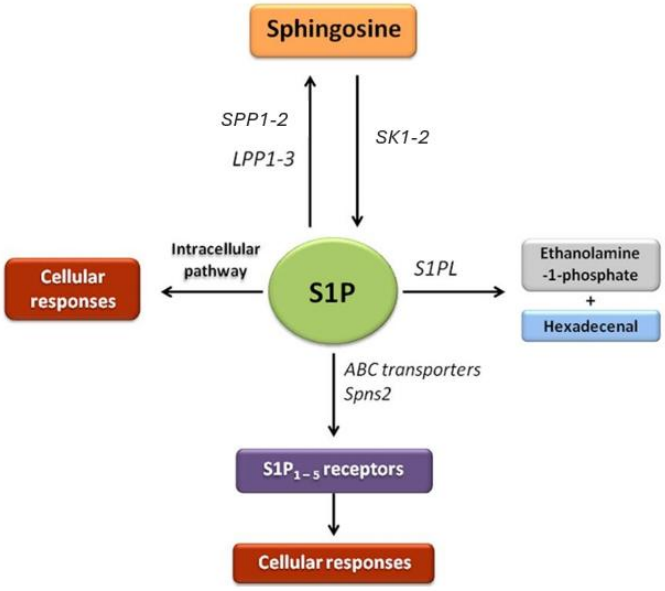

Figure 1-13. Brief overview of S1P metabolism and signaling (González-Fernández et al. 2017)

While the extracellular roles of S1P are well established, recent studies have uncovered several intracellular targets. S1P has been shown to interact with and regulate key signaling mediators, including the chromatin-modifying enzyme histone deacetylase-1 (HDAC-1) (Hait et al. 2009), TNFα receptor-associated factor 2 (TRAF-2) (Alvarez et al. 2010), the mitochondrial regulator prohibitin-2 (Strub et al. 2011), atypical protein kinase-C (Kajimoto et al. 2019), and the catalytic subunit of telomerase reverse transcriptase (Panneer Selvam et al. 2015). S1P promotes cell survival and proliferation, whereas Cer functions as a pro-apoptotic mediator. Sph, generated from Cer via ceramidase, can also trigger apoptosis in various cell types. However, Sph can be phosphorylated into S1P, which counteracts Cer-induced apoptosis (Cuvillier 2002). This interplay, known as the sphingolipid rheostat, determines cell fate by balancing levels of S1P and Cer (Kroll et al. 2020) (Figure 1-14). Since this equilibrium is regulated by enzymes responsible for sphingolipid metabolism, targeting these enzymes has been proposed as a potential therapeutic approach in cancer (Cuvillier et al. 2010). Studies have identified that S1P plays a pivotal role in the ezrin, radixin, and moesin (ERM) regulation (Adada et al. 2014). ERM proteins serve as structural linkers between the actin cytoskeleton and the plasma membrane, while also providing a scaffold for signaling cascades that regulate cell proliferation, migration, invasion, and division (Bretscher et al. 2002). Cer and S1P have opposite effects as S1P leads to activation through phosphorylation, whereas ceramide induces ERM deactivation via dephosphorylation (Adada et al. 2014).

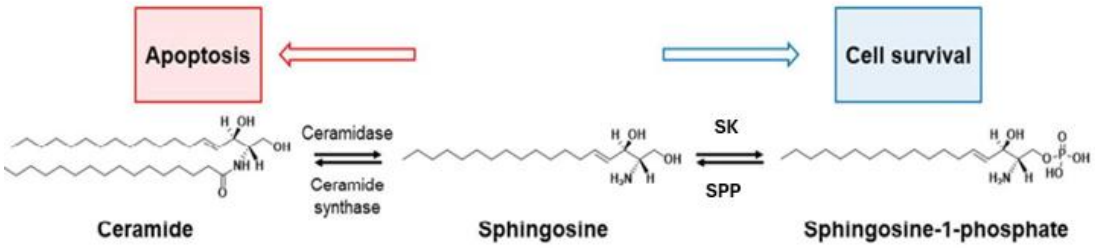

Figure 1-14**.** The sphingolipid rheostat. SK, sphingosine kinase; SPP, S1P phosphatase (Kroll et al. 2020)

Plasma (~1 μM) and lymph (~100 nM) contain much higher concentrations of S1P compared to the interstitial fluid of tissues, where S1P is rapidly degraded (Spiegel and Milstien 2011; Richard L. Proia and Hla 2015). This concentration gradient is essential for extracellular

S1P functions, including lymphocyte egress and vascular barrier regulation (Olivera et al. 2013). To sustain this gradient, intracellularly produced S1P must be actively secreted. Multiple *in vitro* studies have shown that ATP-binding cassette (ABC) transporters contribute to S1P export (Olivera et al. 2013; Kim et al. 2009). These transporters contain two transmembrane domains with six α-helical segments that form a passage for substrate transport, along with two cytosolic ATP-binding cassettes. In humans, 49 ABC genes are classified into seven subfamilies (ABCA–ABCG) (Kim et al. 2009). Specific roles in S1P export have been reported: ABCC1 in mast cells (Mitra et al. 2006), ABCA1 in astrocytes (Sato et al. 2007) and platelets (Kobayashi et al. 2006), and both ABCA1 and ABCC1 in endothelial cells (Lee et al. 2007). Collectively, these findings highlight ABC transporters as important mediators of S1P release. More recently, a dedicated S1P transporter, Spns2, was identified in zebrafish as an essential gene for heart precursor migration during embryogenesis (Kawahara et al. 2009). Spns2 was the first mammalian S1P transporter to be identified. It plays a crucial role in the lymphatic system by providing S1P to the lymph and facilitating lymphocyte circulation (Chen et al. 2023). Spns2, a member of the Spinster-like family of transmembrane proteins, plays a crucial role in S1P export. *In vivo* studies showed that deletion of Spns2 in mice reduces plasma S1P levels around 50% and impairs lymphocyte egress (Fukuhara et al. 2012). In recent years, the major facilitator superfamily transporter 2b (Mfsd2b) was identified as responsible for exporting S1P from red blood cells and platelets; knockout of Mfsd2b in mice also reduced plasma S1P levels by approximately 50% (Vu et al. 2017).

High plasma S1P concentrations are mainly maintained by red blood cells and endothelial cells, both of which are specialized for high extracellular S1P production. Since S1P has a lipophilic nature, it needs to be gathered with aqueous-soluble compounds for transportation in the plasma and to produce paracrine and endocrine effects. In plasma, most S1P is bound to protein carriers, primarily HDL (~60%) and albumin (~30%), with smaller fractions associated with VLDL and LDL (Argraves and Argraves 2007) . Binding to HDL occurs via ApoM, which acts as an S1P chaperone, regulating its bioavailability, protecting it from degradation, and facilitating receptor presentation (Christoffersen et al. 2011). Red blood cells represent the dominant source, harboring approximately half of the circulating S1P, with strong SK1 activity and absence of SPL (Olivera et al. 2013; Hänel et al. 2007). Endothelial cells also play a pivotal role, supported by their dynamic S1P metabolism, which includes high expression of both synthetic enzymes (SKs) and degradative enzymes (SPL, SPPs), and their export capacity via Spns2 (Olivera et al. 2013). Platelets share similar features with red blood cells, high SK

activity and no SPL, allowing them to store S1P. While they contribute little to basal plasma S1P levels, they release significant amounts during platelet activation and clot formation (Figure 1-15) (Olivera et al. 2013; Richard L. Proia and Hla 2015). In lymph, the main S1P source is lymphatic endothelial cells (Pham et al. 2010).

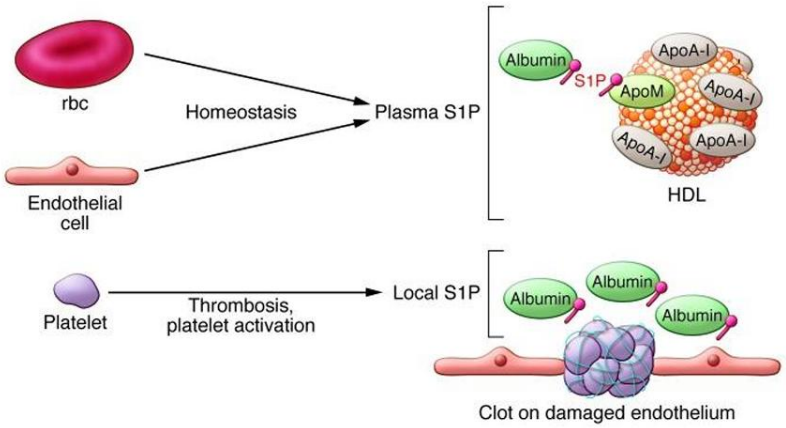

Figure 1-15. Cellular sources of plasma S1P (Richard L. Proia and Hla 2015)

1.6.2 S1P metabolic enzymes

The balance between synthesis of S1P by SKs and its dephosphorylation or irreversible degradation by phosphatases or SPL respectively keeps S1P levels tightly regulated in both a spatial and temporal manner (Figure 1-16) (Spiegel and Milstien 2003). SKs represent a newly recognized group of lipid kinases characterized by evolutionarily conserved domains. Homologs have been identified across species, including worms, flies, humans, mice, yeast, and plants. In mammals, two isozymes, sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2), have been described (Liu et al. 2002). These enzymes share substantial sequence similarity, as most of the SK1 sequence is contained within the longer SK2 sequence, showing 47% and 43% amino acid identity in the N- and C-terminal regions, respectively (Pyne et al. 2016). SKs are broadly expressed and active across all human tissues, yet SK1 and SK2 display distinct developmental expression patterns, adult tissue distribution, and subcellular localization, pointing to different physiological functions (Chan and Pitson 2013). Evidence suggests that the two isozymes may even exert opposing effects: SK1 is generally associated with promoting cell survival and proliferation (Hannun and Obeid 2008; Pyne and Pyne 2010), whereas SK2 has been reported to exhibit pro-apoptotic activity in some contexts (Hofmann et al. 2008; Maceyka et al. 2005; Okada et al. 2005). Conversely, other studies show that SK2 depletion enhances apoptosis and increases chemotherapeutic sensitivity in various cancer cell

types, implying an anti-apoptotic role for SK2 (Weigert et al. 2009; Nemoto et al. 2009; Sankala et al. 2007). Mice with a double knockout of SK1 and SK2 exhibit embryonic lethality due to severe defects in neurogenesis and angiogenesis. In contrast, single knockouts of either SK1 or SK2 display no obvious abnormalities, indicating a degree of functional redundancy between the two enzymes (Chan and Pitson 2013).

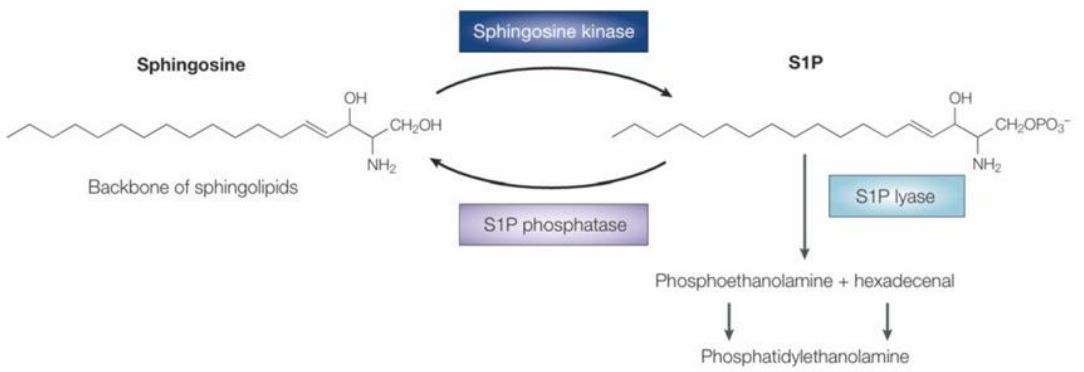

Figure 1-16. Representation of molecular S1P synthesis and degradation (Spiegel and Milstien 2003)

In abnormal conditions, the basal activity of SK1 that help to regulate the relative levels of Sph, Cer and S1P in the cell different, can rapidly be modified in response to agonists such as TNFα, IL-1β, PDGF, EGF and NGF (Rius et al. 1997; Pébay et al. 2005; Mastrandrea et al. 2005; Xia et al. 2002). One of the major post-translational modifications that activates SK1 is its phosphorylation at Ser225 by extracellular signal-regulated kinases 1/2 (1/2), which induces a 14-fold increase in enzymatic activity without affecting its affinity for either ATP or Sph (Pitson 2003). This phosphorylation event is usually transient, as it is counteracted by protein phosphatase 2A (PP2A), which dephosphorylates Ser225 (Barr et al. 2008). Under basal conditions, SK1 is predominantly localized in the cytoplasm. However, phosphorylation by ERK1/2 promotes its translocation to the plasma membrane (Pitson 2003). It has been shown that this translocation can be mediated by calcium- and integrin-binding protein 1 (CIB1), that translocates to the plasma membrane in a process dependent on both its myristoylation and calcium binding (Jarman et al. 2010). Although phosphorylation-driven activation of SK1 has been extensively characterized, it does not represent the sole mechanism of post-translational regulation. Indeed, as it is shown in figure 1-17 several studies have demonstrated that SK1 activity can also be activated through its direct interaction with different regulatory proteins for example: Lyn and Fyn the two Src family protein tyrosine kinases

(PTKs), δ catenin and eukaryotic elongation factor 1A (eEF1A), TRAF2 (an adaptor protein associated with TNFα receptor 1) (Olivera et al. 2006; Leclercq et al. 2008; Fujita et al. 2004; Alvarez et al. 2010). Certain proteins can negatively regulate SK1 through direct interaction, potentially serving to prevent inappropriate cellular production and accumulation of S1P (Chan and Pitson 2013). Notably, FHL-2 and PECAM-1 have been identified as SK1 inhibitors in cardiomyocytes and vascular endothelial cells, respectively. Following extracellular stimulation, SK1 dissociates from these inhibitory complexes, leading to enhanced enzymatic activity (Fukuda et al. 2004; Hayashi et al. 2009).

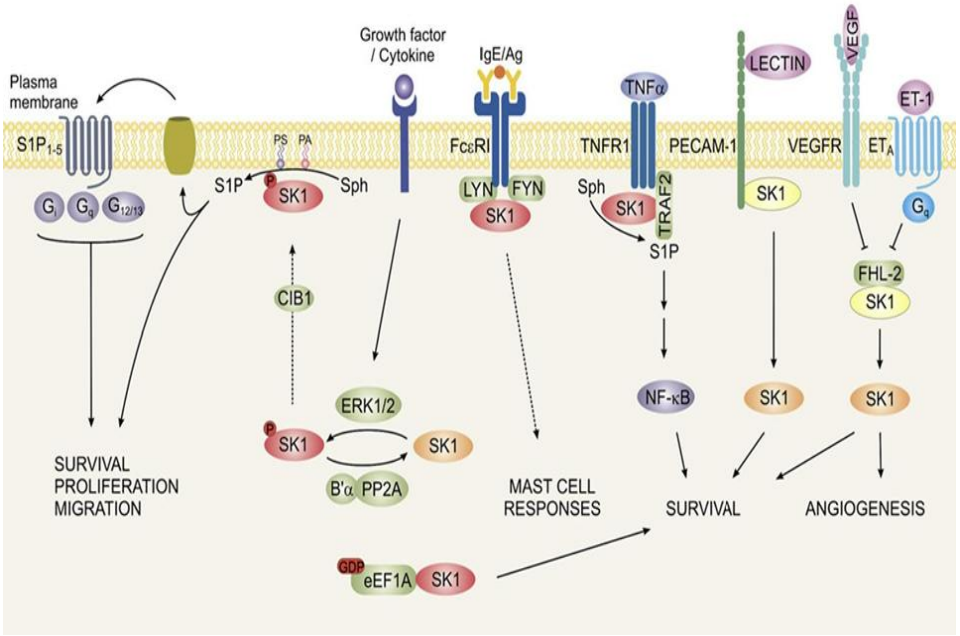

Figure 1-17. Post translational regulation of the metabolic enzyme, SK1 (Chan and Pitson 2013)

The catalytic activity of SK2 can be rapidly enhanced in response to various external stimuli, and like SK1, it is also regulated through phosphorylation (Chan and Pitson 2013). Unlike SK1, SK2 lacks the Ser225 phosphorylation site critical for SK1 activation; however, ERK1/2 still phosphorylates SK2, leading to increased enzymatic activity. Specifically, ERK1/2 phosphorylates SK2 at Ser351 and Thr578 (Hait et al. 2007). Under basal conditions, SK2 is predominantly localized in the nucleus and cytoplasm, though its distribution can shift depending on cellular context for example, SK2 accumulates in the endoplasmic reticulum (ER) during serum starvation and decreases in the nucleus following protein kinase C (PKC)

activation (Figure 1-18) (Hait et al. 2007). Furthermore, several proteins known to interact with SK1 have also been shown to bind SK2, such as Lyn and Fyn and eEF1A (Olivera et al. 2006; Leclercq et al. 2008).

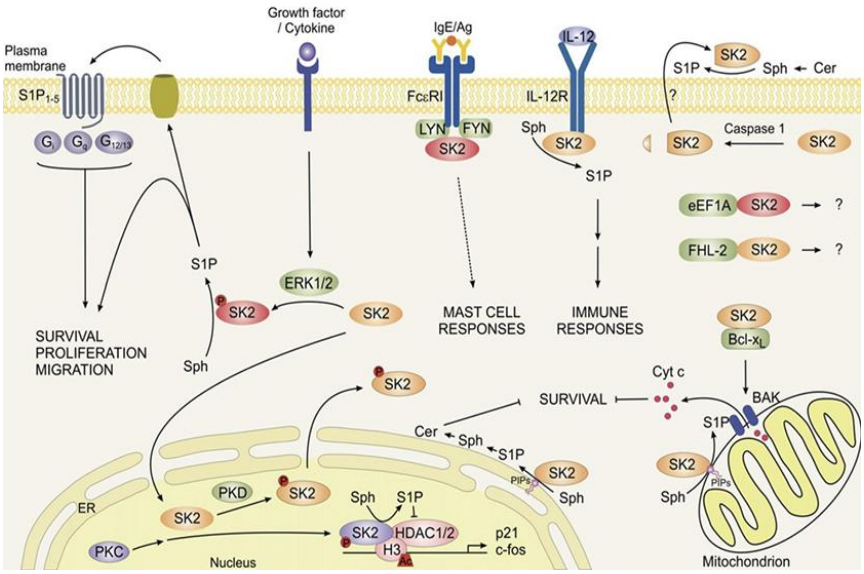

Figure 1-18. Post translational regulation of the metabolic enzyme, SK2 (Chan and Pitson 2013)

S1P degradation occurs either through dephosphorylation by specific phosphatases or via irreversible cleavage by SPL. The first SPPs were identified in yeast, and subsequent homology studies led to the discovery of two mammalian isoforms: SPP1 and SPP2 (Ogawa et al. 2003; Le Stunff et al. 2002). Both enzymes localize to the ER, where they reduce S1P levels by catalyzing its dephosphorylation. Overexpression of SPPs promotes apoptosis by enhancing Cer formation (Le Stunff et al. 2002). Despite their shared function, they differ in expression patterns: SPP1 is ubiquitously expressed, with the highest levels in placenta and kidney and lowest in peripheral blood and small intestine, whereas SPP2 shows a more tissue-restricted expression profile. The two enzymes also differ in their tissue distribution. SPP1 is expressed ubiquitously, showing the highest levels in kidney and placenta and the lowest in peripheral blood and small intestine. In contrast, SPP2 displays a more tissue-restricted pattern, being detected in brain, heart, colon, kidney, small intestine, and lung, with the highest expression in kidney and heart. Notably, SPP2 is absent in skeletal muscle, thymus, spleen, liver, placenta, and peripheral blood leukocytes (Ogawa et al. 2003).

The terminal enzyme of the sphingolipid degradative pathway is SPL which belongs to the superfamily of pyridoxal 5′-phosphate (PLP)-dependent enzymes. In this role, it regulates

not only endogenous S1P levels but also the metabolic flux of other signaling lipids, such as Sph, SM, and Cer (Xiao 2023). Structurally, SPL is an integral membrane protein composed of an N-terminal lumenal domain within the ER), a transmembrane segment, and a soluble pyridoxal-dependent decarboxylase conserved domain (PDCD), which mediates PLP binding and enzymatic activity. The N-terminal portion is embedded in the ER lumen, whereas the large hydrophilic domain harbouring the active site faces the cytosol (Ikeda et al. 2004). SPL catalyzes the irreversible cleavage of S1P and other phosphorylated long-chain bases, producing ethanolamine phosphate (EP) and a long-chain aldehyde. In the case of S1P, the reaction generates hexadecenal (Hex) (Figure 1-19) (Oskouian and Saba 2007). This process requires pyridoxal 5′-phosphate (PLP) as a cofactor and involves the cleavage of the long-chain base at the C2–C3 bond SPL is ubiquitously expressed in mammalian tissues, and its gene is highly conserved from yeast to humans (Xiao 2023; Zhou and Saba 1998). In mammals, SPL is present in most cell types except for erythrocytes and platelets (Ikeda et al. 2004).

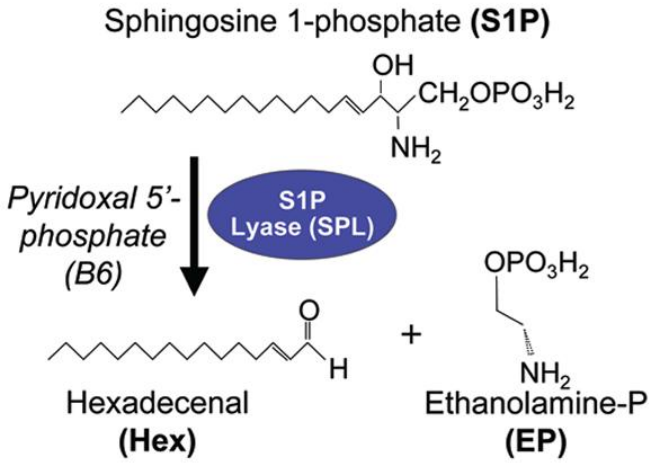

Figure 1-19. Biochemical catalysis of S1P (Oskouian and Saba 2007)

1.6.3 G-protein coupled receptors (GPCR) signaling

Since the initial discovery that S1P functions as the ligand for the previously orphan GPCR endothelial differentiation gene 1 (EDG1) (Lee et al. 1998), it has become evident that S1P plays a key role in regulating cellular activity through autocrine and paracrine signaling by binding to a family of GPCRs. To date, five S1P receptors have been identified: EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, EDG6/$S1P_4$, and EDG8/$S1P_5$ (Spiegel and Milstien 2003). These receptors are coupled to different G proteins, enabling the activation of a wide range of

downstream signaling pathways (Figure 1-20). This receptor diversity allows S1P to exert highly specific physiological effects depending on the relative expression levels of S1P receptors and associated G proteins (Spiegel and Milstien 2003). The expression of the five receptors is tissue- and context-dependent, varying with development and ageing. Among them, $S1P_1$, $S1P_2$, and $S1P_3$ are broadly expressed, whereas $S1P_4$ and $S1P_5$ display more restricted patterns, with $S1P_4$ primarily found in lymphoid and hematopoietic tissues and $S1P_5$ predominantly expressed in the nervous system (Blaho and Hla 2014).

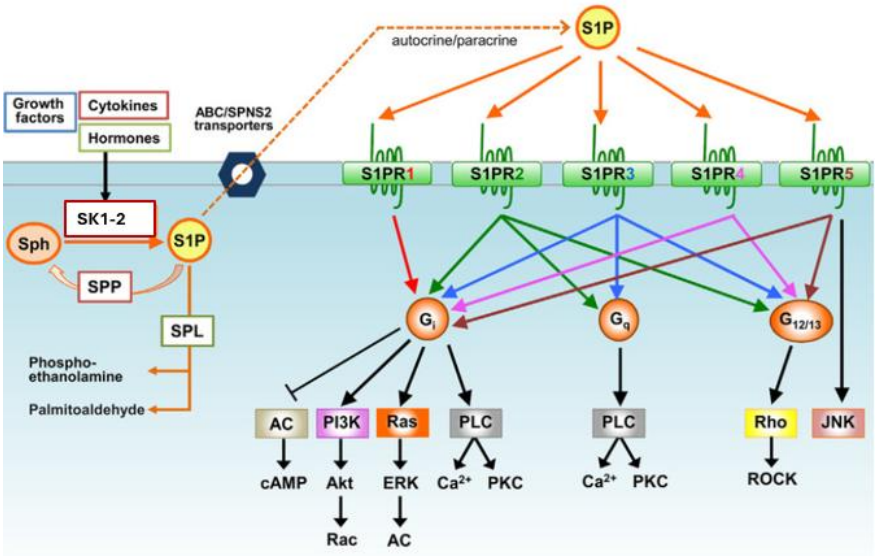

Figure 1-20. S1P/S1PRs signaling pathways (Matsuzaki et al. 2020)

$S1P_1$, originally identified as EDG1, was first discovered in human umbilical vein endothelial cells (HUVEC) (Hla and Maciag 1990) and it was the first S1P receptor to be functionally identified (Lee et al. 1998). $S1P_1$ couples exclusively to the heterotrimeric G protein Gi/o, in contrast to the other S1P receptor subtypes that interact with multiple G proteins (Spiegel and Milstien 2003). Constitutive loss of $S1P_1$ leads to embryonic lethality in mice due to incomplete vascular maturation caused by the absence of vascular smooth muscle cells and pericytes (Liu et al. 2000), and also results in severe neurogenesis defects (Mizugishi et al. 2005). Functionally, $S1P_1$ activates the phosphatidylinositol 3-kinase (PI3K)/Rac pathway in a Gαi-dependent manner to drive cytoskeletal remodeling, adherens junction assembly, and cell migration in vascular endothelial cells (Lee et al. 1999; O'Sullivan and Dev 2013). In addition, this receptor engages Ras-family GTPases and ERK to promote

proliferation, while activation of the PI3K/AKT pathway suppresses apoptosis (O'Sullivan and Dev 2013). Collectively, these signaling outputs support the view that $S1P_1$ is a pro-tumorigenic factor fostering migration, invasion, proliferation, and angiogenesis across several cancer types (P. Wang et al. 2019). Besides its vascular and tumorigenic roles, $S1P_1$ is essential for immune regulation, particularly in controlling lymphocyte trafficking. In mice lacking $S1P_1$ in hematopoietic cells, T cells fail to reach peripheral tissues (Matloubian et al. 2004). $S1P_1$ expression is upregulated during thymic T-cell development to enable egress, but downregulated upon peripheral activation, causing lymphocyte retention in secondary lymphoid organs (Matloubian et al. 2004), with surface $S1P_1$ expression serving as a key determinant of lymphocyte exit kinetics (Thangada et al. 2010). This biology underpins the clinical use of FTY720 (Fingolimod), a functional antagonist of $S1P_1$ that was approved by the U.S. Food and Drug Administration as the first oral therapy for relapsing-remitting multiple sclerosis (Brinkmann et al. 2010). Fingolimod acts as an agonist on all S1P receptors except $S1P_2$ at picomolar–nanomolar concentrations, but induces internalization and degradation of $S1P_1$, leading to lymphocyte sequestration in lymphoid tissues and immunosuppression (Blaho and Hla 2014).

$S1P_2$, initially identified as EDG5, was first discovered in the brain and vascular smooth muscle cells of a rat model (Okazaki et al. 1993; Lado et al. 1994). Later studies revealed its broader expression, with $S1P_2$ mRNA detected in the brain, heart, lung, gastrointestinal tract, liver, kidneys, uterus and testes (Adada et al. 2013). Functionally, $S1P_2$ can couple to Gi/o, Gq, and $G_{12/13}$, though it couples most efficiently to $G_{12/13}$ and strongly activates the small GTPase Rho (Okamoto et al. 2000). Unlike $S1P_1$, which generally promotes proliferation and migration, $S1P_2$ often acts in opposition by regulating S1P-induced cell proliferation, motility, and transcriptional activity (Skoura and Hla 2009). While $S1P_2$ deletion alone does not result in severe phenotypic abnormalities or embryonic lethality, $S1P_1$/ $S1P_2$ double-null embryos exhibit more pronounced vascular defects than $S1P_1$-null embryos, underscoring $S1P_2$'s role in embryonic vascular development (Kono et al. 2004). In addition, $S1P_2$-null mice are deaf due to vascular malformations in the stria vascularis of the inner ear and sensory hair cell alterations in the organ of Corti (Kono et al. 2007). At the vascular level, $S1P_2$ is a key regulator of endothelial barrier function: by coupling to the Rho/ROCK pathway, it activates PTEN phosphatase, which in turn inhibits PI3K signaling, leading to the disruption of adherens junctions and barrier integrity (Sanchez et al. 2007). $S1P_2$ has also been implicated in pathological angiogenesis, since it promotes abnormal retinal vascularization in hypoxia-

induced retinopathy while suppressing normal vascularization (Sanchez et al. 2007). In the liver, $S1P_2$ influences hepatic wound healing, fibrosis, and regeneration; notably, $S1P_2$-/- mice display reduced fibrosis and enhanced regenerative capacity after carbon tetrachloride-induced injury (Ikeda et al. 2009). Interestingly, the role of $S1P_2$ in cell migration is variable as it mediates pro-migratory effects of S1P in breast cancer cells (W. Wang et al. 2019), lung fibroblasts (Hashimoto et al. 2008), and macrophages during cholestatic liver injury (Yang et al. 2015). On the other hand, it has been shown that $S1P_2$ inhibit migration in myoblasts (Becciolini et al. 2006), satellite cells (Calise et al. 2012), basophilic leukemia cells (Yokoo 2004), and thyroid cancer cells (Asghar et al. 2012). Moreover, $S1P_2$, together with $S1P_3$, contributes to S1P-induced Ca^{2+} mobilization (Meacci et al. 2002) and exerts antiproliferative effects by promoting differentiation of myoblasts (Donati et al. 2005).

$S1P_3$, previously known as EDG-3, can couple with $G_{i/o,}$ G_q, and $G_{12/13}$ (Chun et al. 2010). but most efficiently couples to G_q, thereby stimulating the hydrolysis of phosphatidylinositol bisphosphate to generate inositol 1,4,5-trisphosphate, leading to intracellular Ca^{2+} release and activation of PKC (Chun et al. 2010). Functionally, $S1P_3$ is strongly implicated in inflammation, as it promotes the release of inflammatory factors in macrophages (Heo and Im 2019), gingival epithelial cells (Eskan et al. 2008), endothelial cells (Lin et al. 2007), and astrocytes (Dusaban et al. 2017). Beyond inflammation, $S1P_3$ also regulates proliferation: it drives cell proliferation in mouse embryonic stem cells (Ryu et al. 2014), cardiac progenitor cells (Castaldi et al. 2016), and HUVEC, where it additionally stimulates migration and tube formation through upregulation and activation of VEGFR2 (Jin et al. 2018). Moreover, the receptor supports migration in other contexts, including human brain microvascular endothelial cells (Vézina et al. 2018) and bone marrow mesenchymal stem cells (Li et al. 2009). In vascular regulation, $S1P_3$ exerts dual effects. On one hand, S1P induces vasoconstriction by directly activating $S1P_3$, leading to increased intracellular Ca^{2+} and Rho activation in vascular smooth muscle cells (Murakami et al. 2010). On the other, FTY720-P can mediate vasodilation through $S1P_3$ by stimulating nitric oxide (NO) production in human endothelial cells (Tölle et al. 2005). There is increasing evidence for $S1P_3$ involvement in tissue fibrosis. The receptor mediates fibrotic processes in the heart, muscle, lung, and liver (Fan et al. 2021). In mice overexpressing SK1, $S1P_3$ deletion inhibited cardiac fibrosis (Takuwa et al. 2010), while pharmacological inhibition attenuated the profibrotic response to TGFβ1 in myoblasts, as TGFβ1 promotes transdifferentiation of myoblasts into myofibroblasts through the SK1/$S1P_3$ axis (Cencetti et al. 2010). Similarly, in human lung fibroblasts, S1P receptor agonists acted pro-fibrotically

through $S1P_2$ and $S1P_3$ (Sobel et al. 2013), and $S1P_3$ knockout reduced inflammation and fibrosis in bleomycin-induced lung injury models (Murakami et al. 2014). In the liver, S1P promoted bone marrow mesenchymal stem cell migration via $S1P_3$ (Li et al. 2009) and was synthesized by hepatocytes in response to palmitate, subsequently activating fibrotic responses in hepatic stellate cells (Al Fadel et al. 2016). In uterine adenocarcinoma cells, S1P mediated TGFβ1-induced profibrotic activity via $S1P_2$ and $S1P_3$, while also enhancing fibrotic markers independently through $S1P_{1/2/3}$ (Bernacchioni, Capezzuoli, et al. 2021). Moreover $S1P_3$ have a prominent role in the pro-invasive behavior of endometriotic epithelial cells stimulated by neuropeptide S (Prisinzano et al. 2024).

$S1P_4$, originally identified as EDG6, was primarily known to be expressed in lymphoid tissues, including the thymus, bone marrow, spleen, and peripheral leukocytes (Chun et al. 2010). Functionally, $S1P_4$ couples to Gi and $G_{12/13}$ proteins and plays an important role in immune regulation. In mouse T cells, $S1P_4$ signaling was shown to inhibit proliferation and the secretion of various cytokines while promoting the release of the immunosuppressive cytokine IL-10, thereby mediating an immunosuppressive effect of S1P (Olesch et al. 2017; Wang et al. 2005). Beyond its immunomodulatory functions, $S1P_4$ activation has been associated with opening of intracellular calcium stores, phospholipase C (PLC) activation, and stimulation of ERK1/2 signaling (Yamazaki et al. 2000). The receptor also promotes cytoskeletal rearrangements through RhoA activation, leading to ROCK- and LIM kinase–mediated cofilin phosphorylation, actin nucleation, actin filament severing, and myosin light chain activation, which together enhance cytoskeletal contractility (Olesch et al. 2017). Interestingly, $S1P_4$ is upregulated in myoblasts following TGFβ1 stimulation. In this context, $S1P_4$ signaling induced ROCK2-dependent PTEN phosphorylation and subsequent inhibition of AKT signaling, thereby promoting cell death in myoblasts (Cencetti et al. 2013).

$S1P_5$, previously known as EDG8, was the last S1P receptor to be cloned. Its tissue distribution is relatively restricted, being found mainly in the brain and spleen (Jaillard et al. 2005). Functionally, $S1P_5$ plays a key role in oligodendrocyte biology. In immature oligodendrocytes, it mediates S1P-induced process retraction via a Rho–ROCK–dependent pathway, whereas in mature oligodendrocytes it promotes cell survival through activation of the AKT pathway (Jaillard et al. 2005). Additionally, the receptor inhibits the migration of oligodendrocyte progenitors (Novgorodov et al. 2007). Other studies revealed that $S1P_5$ is involved in immune cell trafficking: $S1P_5$-deficient mice display reduced NK cell numbers in the periphery and increased accumulation in lymph nodes and bone marrow due to defective

migration (Walzer et al. 2007). $S1P_5$ expression has also been detected in human brain capillary endothelial cells, where its antagonism reduced vascular permeability and monocyte transmigration in an *in vitro* model of the blood–brain barrier, suggesting a role for $S1P_5$ in maintaining immune quiescence of the barrier endothelium (Van Doorn et al. 2012). Moreover, it has been reported that $S1P_5$ may represent a negative mediator for migration; since S1P binding to $S1P_5$ inhibits the proliferation and migration of oesophageal cancer cells (Hu 2010).

1.6.4 Intracellular targets

Depending on the different subcellular localization of the SK1 and/or SK2, here are a various number of intracellular targets for the produced S1P by SKs (Pyne et al. 2016).

Cytoplasmic S1P generated by SK1 exerts several intracellular functions beyond receptor-mediated signaling. One of which is as a cofactor for the E3 ubiquitin ligase activity of tumor necrosis factor receptor–associated factor 2 (TRAF2) (Xia et al. 2002). S1P directly binds the RING domain of TRAF2, enhancing its ligase activity and thereby promoting Lys63-linked polyubiquitination of the protein kinase RIP1, a critical adaptor in the NF-κB pathway that regulates cell survival, inflammation, and immune responses (Alvarez et al. 2010). The physiological relevance of this mechanism is supported by evidence that the interaction of TRAF2 with TRAF-interacting protein (TRIP), which attenuates TRAF2 ligase activity, also diminishes S1P binding to the RING domain (Park et al., 2015). Nevertheless, the exact role of SK1 in TRAF2/NF-κB signaling remains debated due to conflicting data (Pyne et al. 2016). Another cytoplasmic target of S1P is β-site APP cleaving enzyme 1 (BACE1), the principal β-secretase responsible for initiating amyloid-β production. S1P binding enhances BACE1 activity, whereas pharmacological inhibition or knockdown of SK1/SK2 diminishes Aβ generation, and overexpression of S1P-degrading enzymes has the opposite effect (Takasugi et al. 2011). This highlights a link between intracellular S1P metabolism and Alzheimer's disease pathogenesis. In the nucleus, SK2-generated S1P regulates both genome stability and gene expression. S1P directly binds human telomerase reverse transcriptase (hTERT), preventing ubiquitination by the E3 ligase MKRN1 and thereby stabilizing hTERT. Inhibition or depletion of SK2, or mutation of this binding site, reduces hTERT stability, compromises telomere maintenance to enhance cell proliferation and tumor growth (Panneer Selvam et al. 2015). Nuclear S1P also contributes to transcriptional regulation by targeting histone deacetylases (HDACs). SK2-generated S1P forms part of a chromatin-repressor complex containing histone

H3 and HDAC1/2, where S1P binds HDAC1/2 and inhibits their deacetylase activity. This inhibition maintains histone acetylation at specific promoters, such as those of p21 and c-Fos, thereby enhancing gene transcription (Hait et al. 2009). Consistent with this, fibroblasts from SPL–deficient mice display elevated nuclear S1P levels, reduced HDAC activity, and altered Ca^{2+} homeostasis (Ihlefeld et al. 2012).

Within mitochondria, S1P produced by SK2 binds prohibitin 2 (PHB2), an inner mitochondrial membrane protein crucial for mitochondrial integrity. In SphK2-null mice, the PHB2–cytochrome c oxidase interaction is disrupted, leading to impaired respiration. Similarly, depletion of SK2 or PHB2 abrogates oxidative phosphorylation and eliminates cardioprotective preconditioning, indicating that the SK2–S1P–PHB2 axis is essential for cytochrome c oxidase assembly and mitochondrial respiration (Gomez et al. 2011; Strub et al. 2011). Interestingly, SK2-derived S1P also cooperates with the pro-apoptotic protein BAK to promote cytochrome c release, highlighting a dual role in survival versus apoptosis depending on context (Chipuk et al. 2012). S1P also influences transcription through peroxisome proliferator-activated receptor γ (PPARγ). In endothelial cells, S1P enhances PPARγ interaction with its coactivator PGC1β, increasing target gene expression. This pathway supports vascular development, as PPARγ antagonism reduces S1P-driven tube formation *in vitro*, and $SK1^{-/-}SK2^{+/-}$ mice show impaired vascularization (Parham et al. 2015).

SK1/S1P has also been implicated in the regulation of endocytic membrane trafficking (Shen et al. 2014). Alterations in the cholesterol/SM balance of the plasma membrane induce the formation of tubular endocytic invaginations enriched in N-BAR proteins, where SK1 localizes through a curvature-sensitive interaction between its hydrophobic patch and the lipid bilayer. Silencing of SK1 leads to endocytic recycling defects, which can be rescued by reintroduction of wild-type SK1 but not by the hydrophobic patch mutant V268Q (Shen et al. 2014).

1.6.5 S1P and uterine disorders

Endometriosis lacks a defined etiology but altered signaling pathways have been implicated in its pathogenesis. Primarily, Santulli *et al*. showed transcriptional dysregulation of S1P metabolism in both eutopic and ectopic endometrium. They have demonstrated that endometriotic lesions exhibit reduced SPP2 and SPL mRNA expression compared with healthy

endometrium, alongside upregulation of $S1P_1$ and $S1P_2$ in both eutopic and ectopic tissues, and downregulation of $S1P_3$ (Santulli et al. 2012). Subsequent studies revealed that cystic fluid from endometriomas contains higher S1P levels than non-endometriotic cysts, and that S1P stimulation promotes proliferation of endometriotic stromal cells (Yoshino et al. 2019). Moreover, peritoneal fluid from endometriosis patients shows elevated S1P concentrations, and exposure of intraperitoneal macrophages to S1P enhances IL-6 and COX2 expression (Ono et al. 2021). Our group has recently demonstrated a dysregulation of S1P metabolism and signaling in endometriosis. Specifically, $S1P_1$ expression was significantly elevated in OMA, whereas $S1P_3$ and $S1P_5$ were increased in both OMA and DIE. In addition, SK1 and CIB1 levels were higher in OMA and DIE compared to control endometrium (Bernacchioni, Capezzuoli, et al. 2021). Moreover, S1P contributes to TGFβ1-induced fibrosis in an *in vitro* EMT model of uterine adenocarcinoma cells. Inhibition of SKs or $S1P_{2/3}$ effectively blocked the profibrotic effects of TGFβ1. Notably, S1P alone was also able to upregulate fibrotic markers through $S1P_{1/2/3}$ signaling (Bernacchioni, Capezzuoli, et al. 2021). In addition, we have demonstrated that OMA tissues show increased mRNA levels of $S1P_1$, $S1P_3$, and $S1P_5$, receptors known to promote cell proliferation and migration, consistent with their previously reported involvement in the fibrogenesis of various other tissues (Donati et al. 2021). More recently, we demonstrated that the invasive phenotype induced by Neuropeptide S (NPS) in endometriotic epithelial cell lines depends on SK1, SK2, as well as $S1P_1$ and $S1P_3$. Notably, inhibition or silencing of either of the two isoforms of SKs or of these receptors completely abolished the pro-invasive effects of NPS (Prisinzano et al. 2024). Furthermore, the RhoA/Rho kinase pathway, downstream to S1P receptor signaling, was found to be critically implicated in invasion and cytoskeletal remodeling elicited by NPS. *In vivo* studies using surgically induced mouse models of endometriosis demonstrated that pharmacological inhibition of SK1 significantly reduced lesion size and vascularization (Rudzitis-Auth et al. 2021). Moreover, treatment with FTY720 or the selective $S1P_1$ antagonist SEW2871 markedly suppressed lesion growth, fibrosis, and inflammation (Zhang et al. 2023). Collectively, these findings strongly support a critical role for the S1P signaling pathway in the pathogenesis of endometriosis.

Despite high prevalence of uterine fibroids among benign uterine tumors and affecting up to 70% of women of reproductive age, the pathogenesis of leiomyomas remains incompletely understood (Bulun 2013). Recently, attention has turned to the role of sphingolipid signaling in fibroid development and progression. The S1P signaling pathway has been recently identified as markedly altered in uterine fibroids, with significantly elevated expression of the

enzymes SK1 and SK2, as well as the receptors $S1P_2$, $S1P_3$, and $S1P_5$, in leiomyoma tissues compared to the surrounding myometrium (Bernacchioni, Ciarmela, et al. 2021). Raymond *et al*. demonstrated that in ELT3 rat uterine leiomyoma cells, S1P exerts both proliferative and anti-apoptotic effects, with SK1 mediating the anti-apoptotic action of endothelin-1 (Raymond et al. 2006). In the same cell model, S1P was also shown to enhance COX expression via ABCC1-dependent S1P release and subsequent $S1P_2$ activation (Tanfin et al. 2011). Further insights came from a metabolomics study which revealed distinct metabolic profiles across fibroid subtypes defined by genetic alterations, such as MED12 mutations, HMGA2 overexpression, or fumarate hydratase (FH) inactivation. Notably, MED12-mutated fibroids exhibited significantly reduced levels of sphingolipids and phosphatidylserines, underscoring the metabolic and molecular heterogeneity of leiomyomas (Heinonen et al. 2017).

1.7 Endocannabinoid System

1.7.1 Generalities

The Endocannabinoid System (ECS), discovered in 1988, regulates numerous physiological processes including metabolism, mood, motor function, appetite, cardiovascular control, stress response, cell fate, immune and inflammatory response, endocrine function, neurotransmission and pain (Pacher et al. 2006; Di Marzo 2008). Growing evidence links the ECS to various health and disease states, making it a promising pharmacological target for conditions such as pain, mood disorders, obesity, neurodegenerative and cardiovascular diseases, and metabolic disorders. The ECS is composed of endocannabinoids, cannabinoid receptors, and the enzymes responsible for synthesize and degradation of endocannabinoids (Figure 1-21) (Lowe et al. 2021; Devane et al. 1988). Cannabinoids, in general, are very potent anti-inflammatory agents. Cannabinoids have demonstrated the ability to downregulate cytokine and chemokine production and, in doing so, are able to suppress inflammatory responses. As such, both endocannabinoids and phytocannabinoids may be promising tools in the treatment of inflammatory disorders (Nagarkatti et al. 2009). Endogenous cannabinoids are lipid-based molecules that activate cannabinoid receptors, producing effects that partially mimic those of cannabis' psychoactive compound, Δ9-tetrahydrocannabinol (THC). Endocannabinoids are fatty acid derivatives and include N-arachidonoylethanolamide (AEA), N-

palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA) and 2 arachidonoylglycerol (2-AG). Other less studied endocannabinoids include N-arachidonoyl-dopamine (NADA) nolidan ether and virodhamine (Fezza et al. 2014). The two most extensively studied and earliest identified endocannabinoids are anandamide (arachidonoyl ethanolamide) and 2-arachidonoyl glycerol (2-AG). A key characteristic of these molecules is that their precursors are embedded within cell membrane lipids (Lu and Mackie 2016). Under most physiological conditions, 2-AG concentrations are much higher than that of AEA (Fezza et al. 2014).

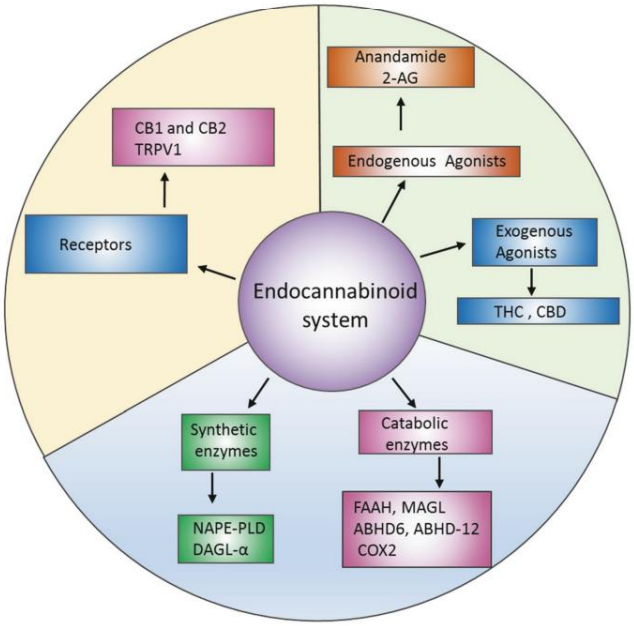

Figure 1-21. Endocannabinoid system and its central components (Ahmadalipour et al. 2024)

N-arachidonoylethanolamide, commonly known as anandamide (AEA), is part of the N-acylethanolamide (NAE) family of endogenous fatty acid amides and is synthesized from arachidonic acid (Fezza et al. 2014). Anandamide was the first endogenous ligand identified for cannabinoid receptors (Di Marzo et al. 2001). Initially discovered in porcine brain, it has been found in various human tissues, including the brain, reproductive organs, immune cells, placenta, fetal membranes, endothelial cells, anterior eye, pituitary gland, and breast tissue (Maccarrone et al. 2015). AEA is involved in several pathological processes and functions as a partial agonist of cannabinoid receptors.

The second most common endogenous ligand for cannabinoid receptors or endocannabinoid to be discovered was 2-arachidonoylglycerol (2-AG) , first identified in 1995 from canine gut (Mechoulam et al. 1998). It is identified as a full agonist of both CB1 and CB2 receptors which is associated with numerous physiological processes, such as inflammation, neuroprotection, pain perception (Savinainen et al. 2001). Similar to AEA, it functions as a retrograde neurotransmitter at both inhibitory and excitatory synapses (Baggelaar et al. 2018). However, its concentration in the brain is approximately 170 times higher than that of AEA. It has been found in various tissues both in human and animals such as central nervous system, brain, immune system and in female reproductive system they have found in the uterus cervix ovaries (Cota 2008; Murataeva et al. 2014; Maia et al. 2020).

1.7.2 Biosynthesis

Endocannabinoids are thought to be synthesized enzymically 'on demand' from lipid precursors residing in the plasma membrane (Di Marzo et al. 1998). There is another hypothesis that states that they are synthesised and stored in intracellular lipid droplets and released from those stores under appropriate conditions, and then transported around the cell by fatty acid binding proteins (Chevaleyre and Castillo 2003). However, another study making the argument that experimental conditions may explain the discrepancies (Hashimotodani et al. 2013). Endocannabinoids can be synthesized through multiple pathways, which relatively depends on tissue type, developmental stage, and disease conditions. AEA is well known to be synthesised through the hydrolysis of N-arachidonoyl phosphatidyl ethanolamine (NAPE) by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo et al. 1994). Several other pathways have also been described, and these may operate in a tissue-specific manner (Leung et al. 2006). The most recognized pathway for generating 2-AG is a two-step process: first removal of the inositol triphosphate from arachidonoyl-containing phosphatidyl inositol bis phosphate (PIP2) and then removal of the acyl group in the 1 position by a diacylglycerol lipase (DAGL) (Kohnz and Nomura 2014). Two isoforms of diacylglycerol , DAGLα and DAGLβ, are abundant in brain however DAGLα is typically more important for synaptic production of 2-AG and DAGL β is more important for microglial formation of 2-AG (Viader et al. 2016; Gao et al. 2010; Tanimura et al. 2010). Disrupted synaptic localization of DAG lipase-α has been linked to neurological disorders. Notably, behavioral and physiological impairments caused by its mislocalization can often be alleviated by inhibiting 2-AG

degradation, suggesting a potential therapeutic strategy that deserves additional investigation (Jung et al. 2012) . Most studies measuring endocannabinoids synthesis and release rely on tissue disruption, extraction, and chromatography followed by mass spectrometry (Piscitelli and Bradshaw 2017). These techniques don't permit sequential observation of the same tissue over time and are limited in spatial resolution. Newly developed and continuously improved fluorescent cannabinoid receptor–based probes for endocannabinoids detection are expected to significantly advance our understanding of where they are synthesized (Jing et al. 2019).

1.7.3 Degradation

AEA is primarily degraded by fatty acid amide hydrolase (FAAH) which converts it into ethanolamide and arachidonic acid. FAAH knockout mice show ~15-fold higher AEA levels, highlighting FAAH's central role in regulating AEA tone (Cravatt et al. 2001). Humans express two FAAH isoforms: FAAH-1, the main hydrolase abundant in the brain, and FAAH-2, present in heart and ovary, with some tissues co-expressing both to regulate distinct or overlapping AEA pools (Maccarrone et al. 2010). Beyond FAAH, AEA can also undergo oxygenation via COX2, lipoxygenases (5, 12, 15-LOX), and cytochrome P450s, producing various bioactive metabolites such as prostaglandin-ethanolamides, hydroxy-anandamides, and epoxyeicosatrienoyl-ethanolamides (Maccarrone 2017). 2-AG is primarily metabolized into arachidonic acid (AA) and glycerol through hydrolysis of its ester bond, a process mainly catalyzed by the presynaptic enzyme monoacylglycerol lipase (MAGL), which accounts for ~85% of 2-AG degradation in the mouse brain, and its deletion can elevate 2-AG levels by up to tenfold (Blankman et al. 2007) (Figure 1-22). Additional enzymes, including α, β-hydrolase domain-containing protein 12 (ABHD12; ~9%), ABHD6 (~4%), FAAH, and the recently identified ABHD2, contribute modestly (<10%) to its catabolism. Beyond hydrolysis, 2-AG can also serve as a substrate for COX, lipoxygenase, and cytochrome P450 (CYP450) enzymes, further reducing its levels and signaling (Simard et al. 2022).

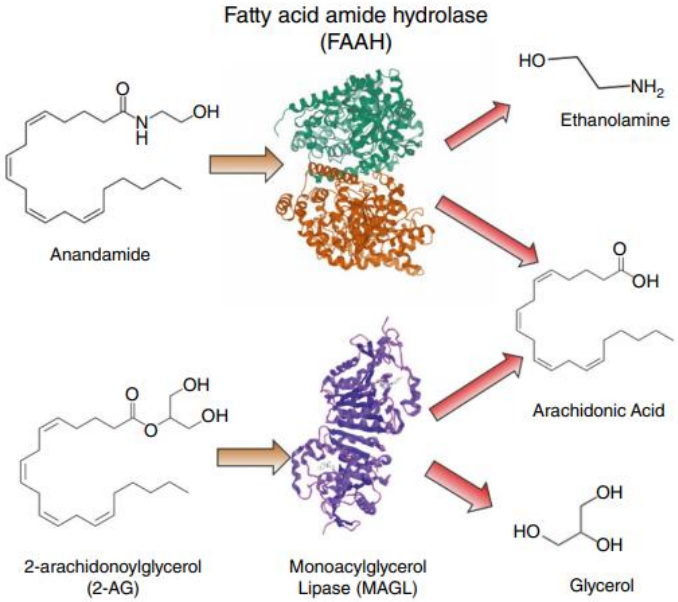

Figure 1-22**.** The two most studied enzymes responsible for catabolism of endocannabinoids (Ahmadalipour et al. 2024)

1.7.4 Cannabinoid Receptors

Several receptors have been identified for endocannabinoids (Figure 1-23). These are the GPCRs and non-GPCR cannabinoid type receptors. CB1 and CB2 are the best-characterized classical GPCRs cannabinoid receptors in which primarily coupling to inhibitory G proteins. They inhibit adenylyl cyclase and certain voltage-sensitive calcium channels, stimulate MAPKs and inwardly rectifying potassium channels , and recruit beta-arrestins, among other actions (Howlett et al. 2002). CB1 receptors are particularly enriched in the nervous system, but also present in certain cells of the immune system, adipose tissue, liver, muscle, reproductive cells, kidney and lungs (Pagotto et al. 2006). The CB1 is found in the rodent uterus and blastocyst where the receptor contributes to normal fetal implantation and implantation failure (Paria et al. 2002). CB2 is primarily expressed in immune and blood cells, and hematopoietic systems but later was found to be present in the brain, nonparenchymal cells of the cirrhotic liver, in the endocrine pancreas and in the bone (Munro et al. 1993; Pacher et al. 2006). Primarily CB1 and CB2 receptors couple to inhibitory G proteins ($G_{i/o}$) and engage the pathways associated with $G_{i/o}$. Under some conditions, cannabinoid receptors can also stimulate cAMP formation and engage $G_{q/11}$ pathways (Lauckner et al. 2005). Like other GPCRs, CB1 and CB2 receptors display functional selectivity, meaning that different ligands can activate distinct signaling pathways. This phenomenon can be explained by the idea that

GPCRs adopt multiple conformations, each with different abilities to interact with intracellular signaling effectors. Depending on the ligand, specific conformations are stabilized, so structurally unrelated agonists may drive very different signaling cascades, leading to diverse biological outcomes (Wootten et al. 2018). Moreover, cannabinoid receptor ligands differ in their intrinsic efficacy. Notably, THC acts as a low-efficacy agonist at CB1, whereas 2-AG and most synthetic CB1 agonists exhibit high efficacy (Deng et al. 2018). Other cannabinoid receptors can be divided into orphan GPCRs or vanilloid type receptors. The first one that binds to cannabinoids is GPR55 and was initially reported to be highly expressed in human striatum (Marchese et al. 1999). The transient vanilloid receptor (TRPV1) is a ligand-gated cation channel activated by various noxious stimuli including heat, acid, as well as endocannabinoids and vanilloid compounds such as capsaicin (Szallasi et al. 2007; Pertwee et al. 2010). TRPV1 is expressed in various tissues, such as, skin, fibroblast, liver, prostate and bladder smooth muscles (Messeguer et al. 2006). Activation of CB receptors initiates multiple signaling pathways, including inhibition of cAMP/PKA cascade and modulation of the MAPK-p38 pathway, as well as phosphorylation of ERK1/2, all of which play key roles in placental endocrine function(Figure 1-24) (Costa et al. 2016).

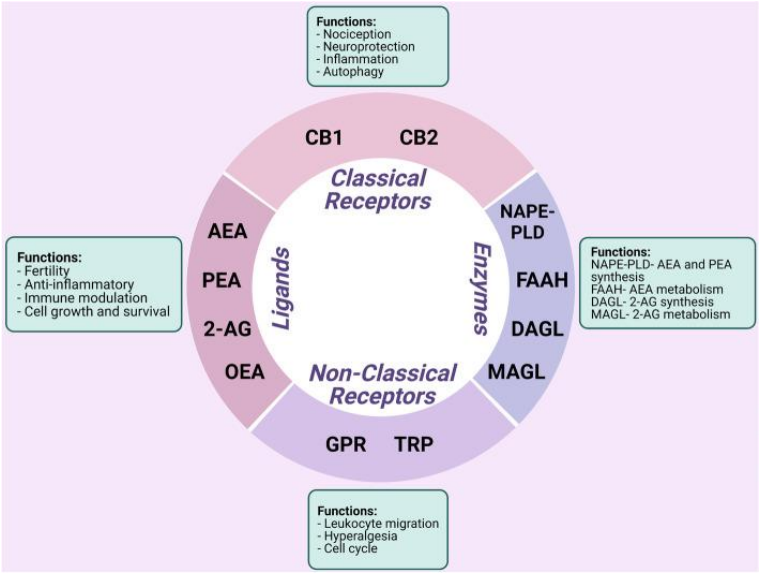

Figure 1-23. Summary of the endocannabinoid system parameters (Lingegowda et al. 2022)

Other non-classical 'orphan' cannabinoid receptors include GPR119 and GPR18. GPR119 has the greatest affinity for OEA and has a more limited tissue distribution, being found predominantly in the pancreas and intestinal tissues (Godlewski et al. 2009). N-

arachidonoyl glycine (NAGLy), the endogenous metabolite of AEA has been reported to be a GPR18 ligand (Kohno et al. 2006). Lately, PPARs have also been considered as receptors for endocannabinoid. PPARs have been shown to be stimulated by endocannabinoids under both physiological and pathological conditions and of interest is the fact that they have a higher affinity for OEA and PEA than the other endocannabinoids (Pistis and Melis 2010).

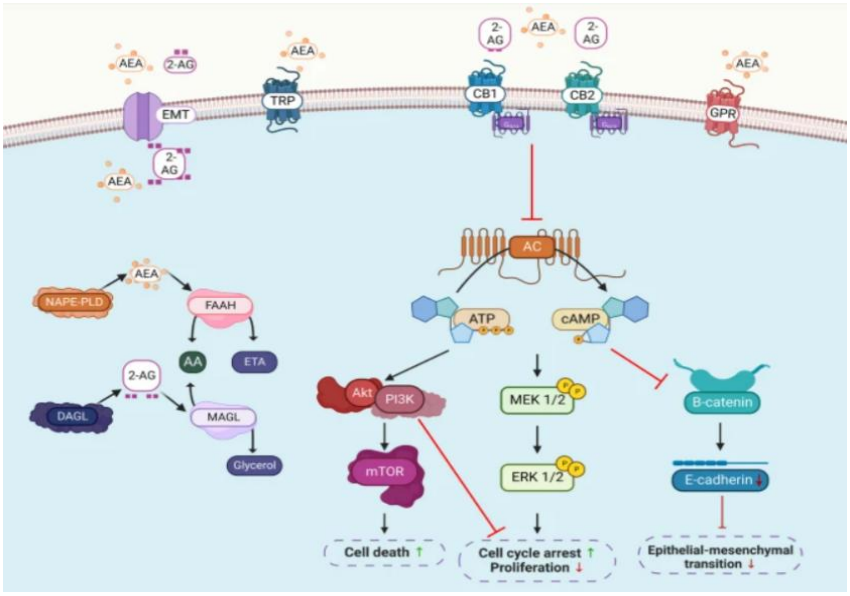

Figure 1-24. The endocannabinoid system signaling cascade (Lingegowda et al. 2022)

1.7.5 Transport

Endocannabinoids, such as AEA and 2-AG, bind to CB1 and CB2 receptors to elicit diverse biological effects. Despite their lipophilic nature, they freely cross cell membranes (Muccioli 2010). Several mechanisms have been proposed to explain their reuptake and signal attenuation, including endocytosis, passive diffusion, AEA membrane transporter (AMT), facilitated diffusion, intracellular sequestration, and transport via intracellular proteins, though none have been definitively proven (Maccarrone et al. 2010). Experimental studies suggest that AEA uptake is temperature-dependent, saturable, and independent of energy sources like ion gradients or ATP hydrolysis (Thors and Fowler 2006). Among the proposed mechanisms, facilitated diffusion is the most widely accepted, supported by extensive research, with extracellular AEA able to cross the plasma membrane along its concentration gradient without carrier proteins (Glaser et al. 2005). Research on 2-AG transport is limited due to its rapid metabolism (Baggelaar et al. 2018). Evidence suggests that 2-AG uptake can be blocked by

inhibitors of AEA transport, indicating a possible shared carrier mechanism (Chicca et al. 2012; Bisogno et al. 2001) .Inside cells, 2-AG has been shown to bind FABP5, though the role of FABP5 or other candidate proteins in regulating 2-AG's physiological functions remains unclear (Sanson et al. 2014).

1.7.6 Endocannabinoids and Endometriosis

Alterations in the ECS have been observed in endometriosis, but its precise contribution to disease onset and progression remains unclear. This area warrants further study, as endocannabinoids regulate key processes such as inflammation, cell proliferation, survival, and migration—all central to endometriosis (Sanchez et al. 2012). ECS elements are highly expressed in human uterus with highest levels of N-acylethanolamines found in endometrium (Scotchie et al. 2015). Cannabinoid receptors and AEA/2-AG metabolic enzymes are present in uterine tissue and fluctuate across the menstrual cycle, with expression levels varying in response to steroid hormone changes (Taylor et al. 2010). The expression of ECS receptors has been previously mentioned in DIE and OMA, reporting significantly higher expression of CB2 in ovaries with endometriotic lesions than in normal ovaries (Allam et al. 2022; Leconte et al. 2010; Taylor et al. 2010). A reduction in FAAH, NAPE-PLD, MAGL, and DAGL has been observed in endometriotic and adenomyotic tissues, suggesting impaired endocannabinoid turnover and contributing to the elevated AEA levels seen in patient plasma (Bilgic et al. 2017). Additionally, TRPV1 mRNA expression is significantly higher in ectopic endometrial tissue from deep infiltrating endometriosis compared to eutopic and healthy endometrium (Bilgic et al. 2017; Bohonyi et al. 2017). One of the central pathways driving lesion proliferation and survival in endometriosis is the MAPK signaling cascade, which is strongly linked to macrophage activity during inflammation. Under physiological conditions, MAPK regulates key cellular functions such as proliferation, differentiation, and apoptosis, either through transcription factor modulation or direct interaction with immune mediators (Zhang and Liu 2002). The ECS has been widely investigated for its role in modulating MAPK family proteins and their disease-related responses. Early studies demonstrated that ECS signaling can activate components of the MAPK cascade, including p38 kinase and ERK1/2, to control cell cycle progression (Wartmann et al. 1995). Importantly, this interaction is highly stimulus-dependent, as the microenvironment determines whether MAPK activation leads to pro-inflammatory or anti-inflammatory outcomes (Demuth and Molleman 2006). MAPK proteins are consistently

upregulated during inflammatory stressors such as oxidative stress, heat shock, and apoptosis (Rajashekhar et al. 2011). This is particularly relevant in endometriosis, where chronic inflammation and lesion growth are hallmark features, and MAPK activity is significantly elevated (Cakmak et al. 2018). A defining feature of endometriosis is inflammation. Cannabinoid receptors are well recognized for their involvement in regulating inflammation, exerting either pro- or anti-inflammatory effects depending on the cellular context and disease state (Turcotte et al. 2016). These contrasting effects highlight the dual role of the ECS in inflammation. CB2 signaling regulates dendritic cell (DC) migration by suppressing matrix metalloproteinase-9 (MMP-9), a macrophage- and DC-derived chemoattractant critical for immune cell trafficking. Elevated MMP-9 levels in the plasma and eutopic/ectopic tissues of endometriosis patients support its link to disease. Normally, CB2 activation reduces MMP-9 secretion, limiting DC migration and lowering pro-inflammatory cytokines such as TNFα, IL-6, IL-2, and IFN-γ (Adhikary et al. 2012; Liu et al. 2015). Interestingly, the phytocannabinoid THC has been shown to inhibit peritoneal macrophage chemotaxis via CB2 activation (Raborn et al. 2008), while Rimonabant, a CB1 inverse agonist, suppresses inflammatory responses in HUVEC (Huang et al. 2010). A study examining CB1 receptor expression in the eutopic endometrium of women with endometriosis reported markedly reduced CB1 mRNA and protein levels compared to healthy controls, regardless of the menstrual cycle phase. This downregulation has been linked to the effects of persistent environmental toxicants and IL-1α, which may disrupt normal CB1 expression by promoting a progesterone-resistant phenotype in affected patients (Resuehr et al. 2012). In a mouse model of endometriosis, MAEA promoted lesion growth and upregulated adhesion- and inflammation-related genes, while CB1 deficiency reduced lesion size and lowered Survivin and N-cadherin expression (A.-M. Sanchez et al. 2016). Numerous studies have extensively reviewed the use of cannabinoids for pain relief in endometriosis and the broader role of the ECS in reproductive disorders (Maia et al. 2020; Bouaziz et al. 2017). Endocannabinoids levels fluctuation also take part in endometriosis-associated pain or even in endometriosis pathogenesis. Women with noncyclic abdominal pain showed consistently elevated 2-AG levels in peritoneal fluid across the menstrual cycle, while those with dysmenorrhea exhibited increased 2-AG and decreased AEA specifically during the proliferative phase. Moreover, 2-AG levels correlated positively with PGE2, and the AEA/2-AG ratio correlated with defensins, indicating a potential connection between the endocannabinoid system and inflammatory pain (Andrieu et al. 2022). The findings suggest that elevated AEA and PEA levels may contribute to pain symptoms in endometriosis. As a result, several clinical trials are investigating PEA, alone or combined with

other anti-inflammatory agents, as a potential therapy for endometriosis-related pain (Bouaziz et al. 2017). Although some studies are preliminary, evidence of ECS alterations in endometriosis supports further exploration of cannabinoid-based therapies. Factors such as tissue- and time-specific mediator availability, hormonal and inflammatory modulation, and environmental influences must be considered (Maia et al. 2020).

1.7.7 Endocannabinoid and S1P

S1P receptors and the cannabinoid receptors CB1 and CB2 are part of the lysolipid GPCR superfamily, sharing about 20% amino acid sequence homology (Sanchez and Hla 2004). This structural similarity allows for potential cross-reactivity of ligands between the two systems. Indeed, Fingolimod, a Sph analogue and immunomodulatory drug, has been shown to interact with both Sph and CB1 receptors (Paugh et al. 2006). After phosphorylation by SK2, Fingolimod binds to S1P receptors (except $S1P_2$) and is used in conditions such as kidney transplantation, autoimmune diseases, and multiple sclerosis. Interestingly, both Fingolimod and Sph act as competitive antagonists at CB1 without affecting CB2 (Paugh et al. 2006).

Additional crosstalk between the endocannabinoid and S1P systems has been observed in the regulation of vascular tone. Both systems are cardioprotective and contribute to cardiovascular regulation. The vascular effects of S1P depend on receptor subtype distribution within specific vessels (Coussin et al. 2002). In rat coronary arteries, both S1P and AEA induce vasorelaxation through CB2 and $S1P_3$ receptors. Blocking CB2 attenuates AEA-mediated relaxation, while $S1P_3$ is required for both AEA- and S1P-induced vasorelaxation. Furthermore, AEA promotes SK1 phosphorylation, and inhibiting SK1 abolishes its vasorelaxant effect (Mair et al. 2010). Evidence also shows that CB1 activation can modulate sphingolipid metabolism, leading to Cer accumulation in various cell types (Velasco et al. 2005). Recently it was shown that activating CB2 pharmacologically counteracted the LPS-induced upregulation of SK1 and SK2 transcription in microglial cells (Standoli et al. 2023). Conversely, S1P signaling was found to modulate the ECS by increasing TRPV1 expression while decreasing CB2 levels in skeletal muscle cells (Standoli et al. 2022). The activation of SK, specifically the SK1/S1P regulatory axis, is essential for mediating the rapid hypotensive response to AEA observed in anesthetized mice (Greig et al. 2019). Notably, $S1P_5$ has been reported to exert a negative regulation of the tumorigenic effect induced by CB2 in

glioblastoma cells: bioluminescence resonance energy transfer analysis highlighted that this S1P receptor subtype strongly and specifically interacts with CB2 (Kim and Ghil 2025).

1.8 Aim of the thesis

This study aims to elucidate the role of the ECS and the S1P signaling pathway in hormone-dependent gynaecological disorders characterized by chronic inflammation and fibrosis, including endometriosis, adenomyosis, and uterine leiomyomas. Specifically, we aim to investigate the expression of endocannabinoid receptors in endometriosis and adenomyosis tissue. In addition, we will study the role of endocannabinoid signaling in endometriosis and its possible crosstalk with S1P signaling axis, focusing on inflammation which plays a crucial role in the initiation and establishment of endometriosis. Morely, we aim to investigate the profibrotic effect of S1P in endometriotic epithelial cells, also to examine the protein expression of $S1P_3$ in endometriotic tissues and its association with fibrosis. The other target of this work is to investigate the role of S1P on cellular proliferation and fibrogenesis through characterisation of the receptors isoform and the downstream signaling pathway involved in leiomyoma compared to myometrial cells.

2 . Paper 1

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$S1P_3$ receptor mediates the proinflammatory effect of the endocannabinoid 2-arachidonoylglycerol in endometriotic epithelial cells.

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2.1 Materials and Methods

2.1.1 Materials

All biochemicals, TRI reagent, cell culture reagents, Nutrient Mixture F-12 Ham (F12), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, Penicillin/Streptomycin, phosphatase inhibitor cocktail, protease inhibitor cocktail, 2-AG, bovine serum albumin (BSA), VPC23019, TY-52156, PF-543, the specific siRNA for SK1, SK2, $S1P_1$, $S1P_2$, $S1P_3$ and the scramble siRNA were purchased from Merck Life Sciences (Burlington, MA, USA). C17-Sph and C17-S1P were obtained from Avanti Polar Lipids (Alabaster,AL, USA). Human endometriotic epithelial 12Z cell line (Cat. No. T0764) and the Applied Cell Extracellular Matrix were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). Bradford protein assay, Tris/Glycine/SDS, the EveryBlot Blocking Buffer, the Clarity western ECL substrate and the trans-blot turbo PVDF membrane were obtained from Bio-Rad (Hercules, CA, USA). Anti-$S1P_3$ antibody was obtained from Abcam

Ltd (Cambridge, UK). Anti-SK1, anti-SK2, anti-phospho-SK2 (Thr578) and anti-phospho-SK1 (Ser225) antibodies were purchased from ECM Biosciences LLC (Versailles, KY, USA). Anti-Spns2 antibody was purchased from FabGennix International Inc. Anti-CB1, anti-CB2 and MAEA were purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and anti-GPR18 from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). Anti-TRPV1 was obtained from OriGENE Technologies Inc (Rockville, MD, USA). RayBio® C-Series Human Inflammation Array C1 were from RayBiotech (Peachtree Corners, Georgia, USA). Anti-GAPDH antibodies and secondary antibodies conjugated to horseradish peroxidase, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AllPrep DNA/RNA FFPE kit were purchased from Quiagen (Hilden, Germany). TaqMan Universal Master Mix II, TaqMan gene expression assays, Lipofectamine RNAiMAX, High-capacity cDNA reverse transcription kit and the UltraVision LP Detection System HRP Polymer and DAB Plus Cromogen were obtained from Thermo Fisher Scientific INC (Waltham, MA, USA).

2.1.2 Sample collection

The gene expression study (n=20) and the immunohistochemistry analysis (n=15) were conducted on endometriotic lesions obtained from independent cohort of patients. Control endometrial samples (n=15) used for gene expression analysis were acquired using diagnostic hysteroscopy procedures. Clinical and imaging investigations were performed to exclude a diagnosis of endometriosis or other uterine disorders in controls. All the samples were histologically characterized. The diagnostic hysteroscopy or surgical procedure for lesion removal were performed during the proliferative phase, and all hormonal treatments had been stopped at least 2-3 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 study protocol was approved by the Institutional Review Board (number 13742) and all patients provided written informed consent.

2.1.3 Cell Culture and treatment

Human endometriotic epithelial 12Z cells were cultured in dishes coated with the extracellular matrix using a 1:1 mixture of DMEM:F12, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin at 37°C in 5% CO_2. For the experiments, cells were serum-starved overnight in a medium supplemented with 1 mg/mL fatty acid-free BSA. When

requested, cells were pre-treated with pharmacological antagonists or an equivalent volume of vehicle (DMSO) for 45 min prior to treatment with 2-AG or the corresponding vehicle (ethanol).

2.1.4 Cell Transfection

Endometriotic epithelial 12Z cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX, according to the manufacturer's instructions (Cencetti et al. 2014; 2019). siRNAs diluted in the mixture of DMEM: F12 were incubated with lipofectamine RNAiMAX at room temperature for 20 minutes and then added to cells to a final concentration of 50 nM, in DMEM: F12 supplemented with 10% FBS. After 30 h cells were serum-starved overnight and stimulated with 10 µM 2-AG.

2.1.5 Quantitative real time PCR (qPCR)

Total RNA was extracted from Formalin-Fixed, Paraffin-Embedded (FFPE) tissue samples using the AllPrep DNA/RNA FFPE kit and from endometriotic epithelial 12Z cells employing TRI-reagent following the manufacturer instructions, and reverse-transcribed using the high-capacity cDNA reverse transcription kit, as directed by the manufacturer. TaqMan gene expression assays were used to quantify target gene mRNAs in triplicate on a CFX96 Touch qPCR Detection System (Bio-Rad, Hercules, CA, USA). The target sequences were amplified alongside the reference gene β-actin (Bernacchioni, Capezzuoli, et al. 2021). The $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods were used to calculate the relative expression of mRNA (Livak and Schmittgen 2001; Schmittgen and Livak 2008).

2.1.6 Immunohistochemistry (IHC)

Tissue samples were fixed with formalin, paraffin embedded and 3 µm sections were suitably sliced from each block and then stained for IHC analysis with specific antibodies against CB1 (1:50), CB2 (1:50) GPR18 (1:50) and TRPV1 (1:50). The procedure started with deparaffinization and rehydration. The slides were heated in microwave at 95-97°C in a citrate buffer (pH 6.0) for a total of 20 min and cooled to room temperature (RT) for antigen retrieval. Then the slides were incubated with the primary antibodies at 4°C overnight. To detect the antigen-antibody complexes the "UltraVision LP Detection System HRP Polymer and DAB Plus Cromogen" kit was used accordingly to the manufacturer's instructions: tissue slices were

covered with the "Primary Antibody Enhancer" 10 min at RT followed by 15 min with the secondary antibody conjugated to the horseradish peroxidase. The bound antibody complexes were stained for 3 min 40 sec with diaminobenzidine and then mounted. The slides were analysed at the Histopathology and Molecular Diagnostics of Careggi University Hospital, Florence. Images were obtained by Optikam PRO6 Digital Camera (C-P6), OPTIKA microscope Italy, B-383PLi.

2.1.7 Western Blot (WB) Analysis

Cells were collected in 50 mM Tris, pH 7.5, 120 mM NaCl, 6 mM EGTA, 1 mM EDTA, 20 mM NaF, 15 mM $Na_4P_2O_7$, 1% Nonidet, with the addition of protease and phosphatase inhibitor cocktail and then incubated at 4°C for 30 min. Cells were centrifuged for 10,000g, 15 min at 4°C, and the supernatant containing protein was collected. Proteins were used to perform SDS/PAGE and WB. PDVF membranes were incubated at 4 °C overnight with the primary antibodies and then with specific secondary antibodies at RT for 1 h. The binding of the antibodies with the specific proteins was detected by chemiluminescence employing Amersham Imager 600 (GE Heathcare, Buckinghamshire, UK).

2.1.8 Human Inflammation Array

Pro-inflammatory cytokine secretion into the media was quantified by RayBio® C-Series Human Inflammation Array C1. Endometriotic epithelial cells were cultured and treated with 2-AG or MAEA for 24 h before conditioned media were collected according to the manufacturer's instructions. After the incubation of the media with the antibody arrays membranes, chemiluminescence signals were obtained employing Amersham Imager 600. Signal intensities were used to quantify inflammatory factors secretion using ImageJ software.

2.1.9 Sphingosine kinase in cell Assay

Endometriotic epithelial cells were plated and upon reaching 70% confluence, were serum-starved overnight. Cells were incubated with the exogenous substrate C17-Sph (5 µM) and contemporaneously stimulated or not with 10 µM 2-AG for 30 min. Cells were then harvested

in methanol and subsequently added with internal standard for C17-S1P (10 pmol d7 S1P in methanol). Samples were vortexed, precipitated overnight at −80°C, followed by a 5 min centrifuge at 21300 g, 4°C and then the supernatant was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.1.10 Statistical analysis

Statistical analysis was performed with the use of Student's t-test, Mann-Whitney test, One-way or 2-way ANOVA followed by Bonferroni post hoc test. Graphical representations were generated using GraphPad Prism 10.0 (GraphPad Software) (San Diego, CA, USA).

2.2 Results

2.2.1 Cannabinoid receptors and enzymes expression in human endometriotic lesions

We initially determined the expression of CB1, CB2, GPR18 and TRPV1 in endometriotic lesions of different localizations. The real time PCR analysis reported in Figure 2-1A shows that all the investigated receptors are expressed at mRNA levels in the analyzed lesions. Moreover, the expression levels of each receptor were comparable to those observed in endometrial samples from healthy women (Figure 2-1A). The expression of CB1, CB2, GPR18 and TRPV1 was confirmed at protein level, as demonstrated by the representative IHC images shown in Figure 2-1B: the staining of the analyzed receptors is clearly detectable both in stroma and in glands. For all the analyzed receptors, strong immunoreactivity was observed in the glandular epithelium (black arrows), while the stromal cells showed lower signal (red arrows). In addition, the expression of the enzymes involved in 2-AG and AEA synthesis, NAPE-PLD and DAGL respectively, as well as in endocannabinoid catabolism, FAAH and MAGL that degrade 2-AG and AEA, respectively, were demonstrated in endometriotic lesions (Figure 2-1C). In particular, mRNA levels of NAPE-PLD and MAGL were found to be significantly increased in endometriotic lesions compared to healthy endometrial tissue (Figure 2-1C).

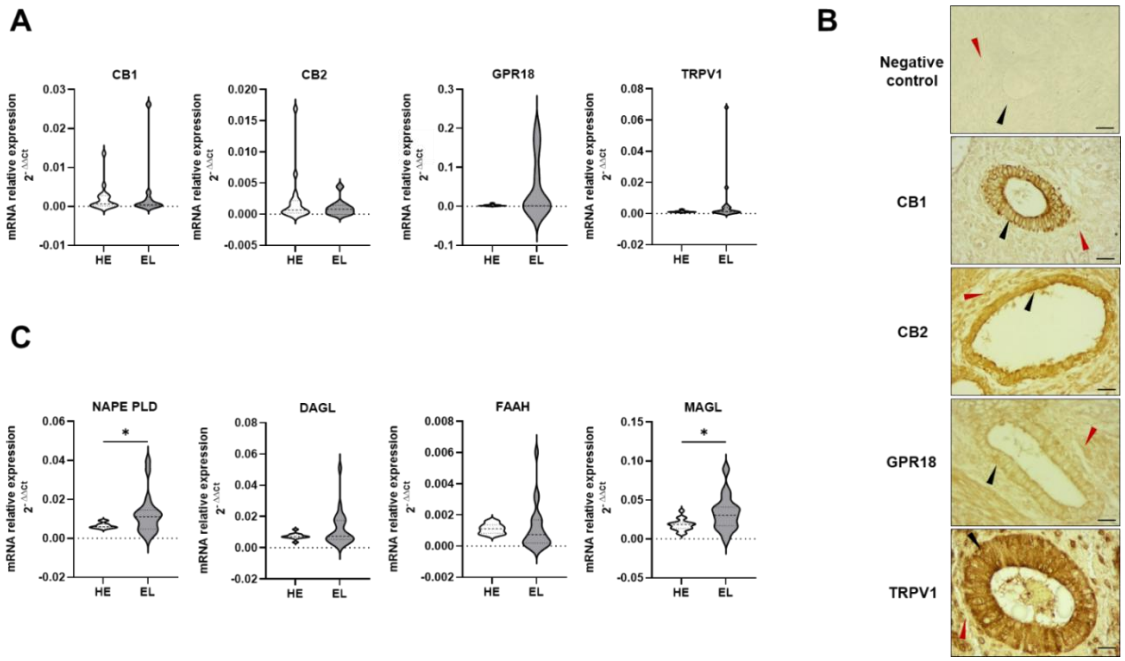

Figure 2-1. **Cannabinoid receptors and enzymes are expressed in human endometriotic lesions**. **(A)** qPCR analysis was performed using TaqMan Gene Expression Assay probes specific for cannabinoid receptors CB1, CB2, GPR18 and TRPV1 in the endometrium of healthy women (HE) (n=15) or endometriotic lesions (EL) of different localization (n=20: 7 ovarian endometriosis and 13 deep infiltrating endometriosis). Results were analyzed employing the $2^{-\Delta Ct}$ method. **(B)** Representative immunohistochemical images of CB1, CB2, GPR18 and TRPV1 expression in endometriotic lesions (n=15: 11 ovarian endometriosis and 4 deep infiltrating endometriosis). The staining with DAB produced a brown precipitate at the site of antibody binding, localized both in epithelial (black arrows) and stromal (red arrows) cells of the endometriotic lesions. The negative control was obtained by processing tissue sections in parallel with the same IHC protocol, omitting the primary antibody. Magnification: ×40, scale bar: 30 μm. **(C)** qPCR analysis was performed using TaqMan Gene Expression Assay probes specific for cannabinoid enzymes NAPE-PLD, DAGL, FAAH and MAGL in the endometrium of healthy women (HE) (n=15) or endometriotic lesions (EL) of different localization (n=20: 7 ovarian endometriosis and 13 deep infiltrating endometriosis). Results were analyzed employing the $2^{-\Delta Ct}$ method.

2.2.2 2-AG induce the pro-inflammatory effect in endometriotic epithelial cells

To characterize the biological action of endocannabinoids in endometriosis, epithelial endometriotic 12Z cells, were employed. We first investigated whether the cells express the endocannabinoid metabolic enzymes and the receptors. qPCR analysis showed that both the biosynthetic (DAGL and NAPE-PLD) and the catabolic (FAAH and MAGL) enzymes were

expressed in endometriotic epithelial cells (Figure S2-1A). Moreover, the expression of the receptors CB1, CB2, GPR18 and TRPV1 was observed at the mRNA level (Figure S2-1A) and further confirmed at the protein level by WB analysis (Figure S2-1B).

Then, we evaluated whether endocannabinoids are able to regulate the inflammatory response in endometriotic epithelial cells. To this aim, the mRNA levels of COX2, interleukin-1β (IL-1β), IL-6 and IL-8 were examined in the cells treated for 24 h with increasing concentrations (2.5 μM, 5 μM, 10 μM) of 2-AG and MAEA, the nonhydrolyzable anandamide analogue. By real time PCR analysis, it was found that 2-AG potently increased the expression of COX2, IL-1β, IL-6 and IL-8 with a maximal effect at 10 μM (Figure 2-2A). On the contrary, the treatment with MAEA did not significantly affect the expression of the analyzed inflammatory factors at any of the tested concentrations (Figure 2-2A).

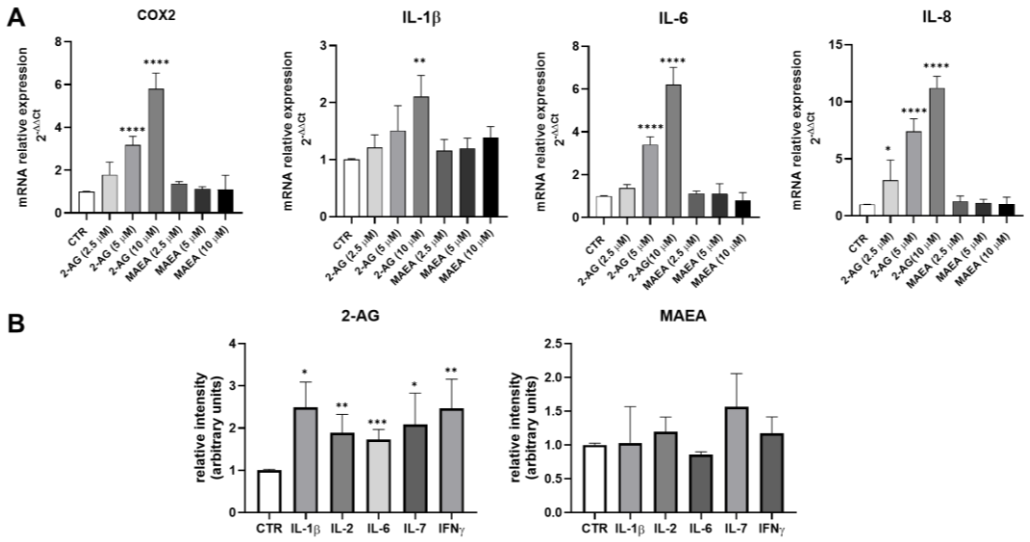

Figure 2-2. **2-AG, but not MAEA, increases the expression and release of different pro-inflammatory factors in endometriotic epithelial cells**. **(A)** Endometriotic epithelial cells were serum-starved for 18 h and treated with increasing concentrations of 2-AG and MAEA for 24 h. mRNA quantitative analysis of COX2, IL-1β, IL-6 and IL-8 was performed by qPCR. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual inflammatory factors of the unchallenged specimen as reference gene. 2-AG increases COX2, IL-1β, IL-6 and IL-8 expression in a statistically significant manner (One-way ANOVA, *p < 0.05; **p < 0.01; ****p < 0.0001). **(B)** Endometriotic epithelial cells were serum-starved for 18 h and treated with 10 μM 2-AG or 2.5 μM MAEA for 24 h. The obtained conditioned media were screened for the content of proinflammatory markers using the Human Inflammation Array as described in the Material and Method section. Results were expressed as fold increase in respect to control. 2-AG induces the extracellular release of IL-1β, IL-2, IL-6, IL-7 and IFNγ in a statistically significant manner (Student's t-test*p < 0.05; **p < 0.01; ***p < 0.001).

To confirm this data, we then examined whether the treatment with the endocannabinoids modulates the extracellular release of functional pro-inflammatory factors. To this aim, the conditioned media obtained from endometriotic cells treated for 24 h with 2-AG or MAEA were analyzed employing an antibody array that allows the simultaneous detection of multiple cytokines. In agreement with the results obtained by real time PCR, 2-AG significantly increased the extracellular release of various pro-inflammatory cytokines, namely IL-1β, IL-2, IL-6, IL-7 and IFNγ (Figure 2-2B). In contrast, MAEA treatment did not significantly affect the extracellular release of these inflammatory factors in 12Z cells (Figure 2-2B).

2.2.3 2-AG modulates S1P signaling axis in endometriotic epithelial cells

Given the key role of S1P in modulating the inflammatory process in endometrial cells (Seidita et al. 2023), we next examined whether cell treatment with 10 μM 2-AG for 24 h regulated the expression of molecules involved in S1P metabolism and signaling. Data shown in Figure 2-3A shows that 2-AG significantly augmented the mRNA levels of SK1, of the receptor isoform $S1P_3$ and of the specific transporter Spns2, suggesting the occurrence of an interplay between 2-AG and S1P signaling in endometriotic cells. In accordance, WB analysis performed in cells treated with 10 μM 2-AG for 24 h showed that $S1P_3$ protein levels were significantly increased by the endocannabinoid (Figure 2-3B). On the contrary, the 2AG-induced increase in mRNA levels was not mirrored by a parallel increase in protein content of SK1 and Spns2, at least at the examined time point (Figure 2-3B). Of note, our group previously reported a significant upregulation of $S1P_3$ both at mRNA and protein levels in endometriotic lesions compared to endometrium from healthy controls (Bernacchioni, Capezzuoli, et al. 2021; Bernacchioni et al. 2024). Interestingly, 2-AG was responsible for a significant increase of CB1 mRNA levels while the expression levels of the other endocannabinoid receptors were not affected (Figure S 2-2).

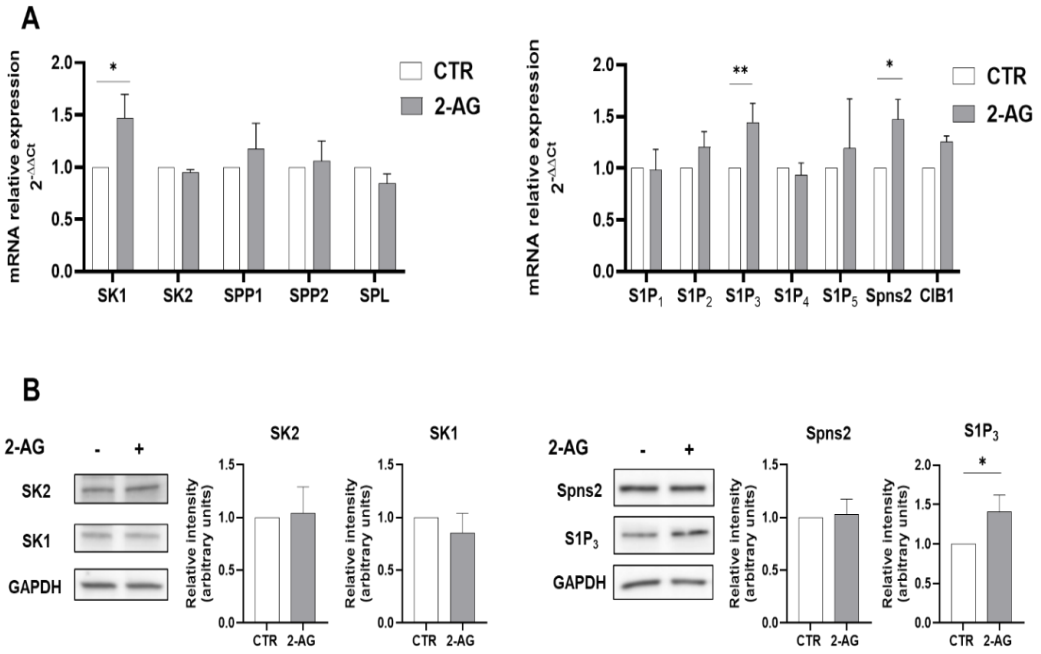

Figure 2-3. **2-AG modulates S1P signaling axis in endometriotic epithelial cells.** Endometriotic epithelial cells were serum-starved for 18 h and treated with 10 μM 2-AG for 24 h. **(A)** mRNA quantitative analysis of S1P metabolic enzymes (SK1, SK2, SPP1, SPP2 and SPL) as well as molecules implicated in S1P signaling ($S1P_{1-5}$, the specific transporter Spns2 and the SK1-activating protein CIB1) was performed by qPCR. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual targets of the unchallenged specimen as reference gene. 2-AG increases SK1, $S1P_3$ and Spns2 mRNA levels in a statistically significant manner (Student's t-test*$p < 0.05$; **$p < 0.01$). **(B)** Protein lysates were analyzed using SDS-PAGE electrophoresis and WB, using specific anti-SK1, anti-SK2, anti-$S1P_3$, anti-Spns2 and anti-GAPDH antibodies. The histograms represent the densitometric analysis of four independent experiments. Data are the mean ± SD and are reported as band intensity normalized to the expression of GAPDH, fold change over control (set as 1). 2-AG increases $S1P_3$ protein content in a statistically significant manner (Student's t-test*$p<0.05$).

2.2.4 2-AG pro-inflammatory action relies on $S1P_3$

Next, the potential role of 2-AG-dependent up-regulation of $S1P_3$ in the pro-inflammatory action of the endocannabinoid was examined. For this purpose, the expression of the pro-inflammatory factors was evaluated in endometriotic cells challenged with 2-AG in the presence or absence of TY-52156 (10 μM), $S1P_3$ specific antagonist, or VPC23019 (10 μM), pharmacological antagonist of $S1P_3$/$S1P_1$. The obtained results show that the enhanced expression of COX2, IL-1β, IL-6 and IL-8 elicited by 2-AG was significantly reduced by pre-incubation with TY-52156 (Figure 2-4A) and VPC23019 (Figure S 2-3), suggesting a crucial role for $S1P_3$ in transmitting the pro-inflammatory action of 2-AG.

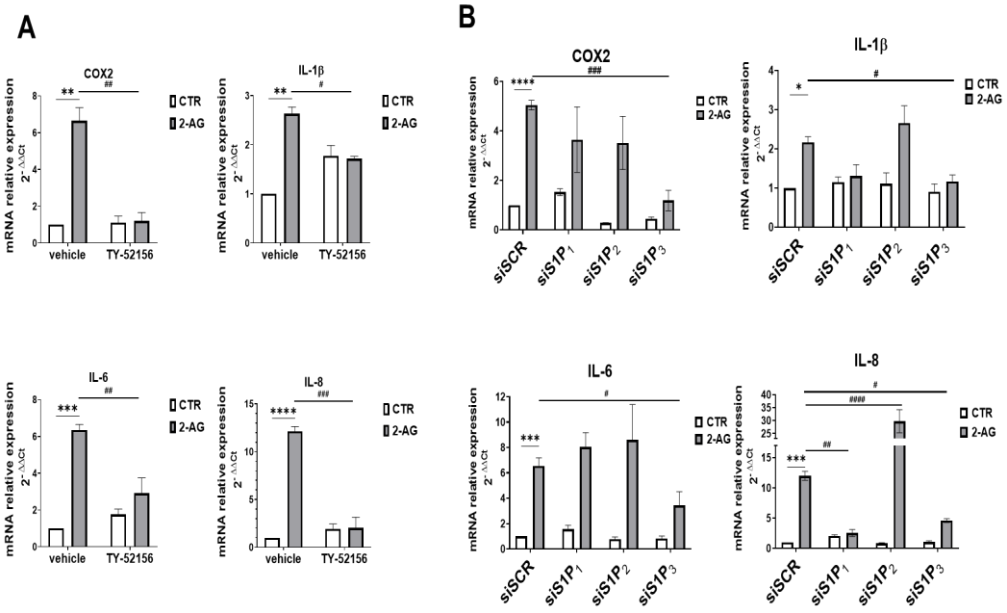

Figure 2-4. **2-AG pro-inflammatory action relies on $S1P_3$ (A)** Serum-starved endometriotic epithelial cells were pretreated or not with the $S1P_3$ antagonist TY-52156 (10 μM) for 45min before being challenged with 10 μM 2-AG for 24 h. mRNA quantitative analysis of COX2, IL-1β, IL-6 and IL-8 was performed by qPCR. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual inflammatory factors of the unchallenged specimen as reference gene. The blockade of $S1P_3$ on 2-AG-induced inflammatory effect was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (#$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$). **(B)** Endometriotic epithelial cells transfected with SCR-, $S1P_1$-, $S1P_2$- and $S1P_3$-siRNA were serum-starved prior to be challenged with 10 μM 2-AG for 24 h. mRNA quantitative analysis of COX2, IL-1β, IL-6 and IL-8 was performed by qPCR. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual inflammatory factors of the unchallenged specimen as reference gene. The effect of $S1P_3$ downregulation in the reduction of 2-AG-induced inflammatory effect was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (#$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$; ####$p < 0.0001$).

To confirm these findings, RNA interference approach was employed to efficaciously reduce the expression levels of $S1P_1$, $S1P_2$ or $S1P_3$ (Figure S 2-4A). Notably, the selective knock-down of $S1P_3$ impaired the 2-AG-induced increase of COX2, IL-1β and IL-6 transcription while the downregulation of the other two receptor isoforms did not alter 2-AG action (Figure 2-4B). Interestingly, the selective knock-down of $S1P_1$ and $S1P_3$ significantly reduced the increase of IL-8 levels elicited by 2-AG, whereas the silencing of $S1P_2$ enhanced the endocannabinoid effect, suggesting positive and negative roles of these receptor isoforms in the specific modulation of IL-8 expression (Figure 2-4B).

2.2.5 2-AG pro-inflammatory action is SK-independent in endometriotic epithelial cells

To gain further insight into the mechanism by which 2-AG exploits S1P signaling to exert its pro-inflammatory effect, we extensively examined the possible involvement of SKs, the enzymes responsible for S1P synthesis. Indeed, on the basis of the S1P "inside-out" mechanism of action, the sphingolipid generated following SK activation acts as ligand of its receptors after being exported outside the cells (Nishi et al. 2014; Blaho and Hla 2014). Since 2-AG treatment was unable to enhance SK protein content (Figure 2-3B), we examined whether the short-term regulation of SK1 and SK2 via their phosphorylation (Pitson 2003; Hait et al. 2007) was implicated in the endocannabinoid action. Data obtained by WB analysis using specific anti-phospho-SK1 or anti-phospho-SK2 antibodies showed that cell challenge with 10 μM 2-AG for different time intervals (5 to 30 min) did not affect SK1 or SK2 phosphorylation, ruling out SK1/SK2 activation by the endocannabinoid (Figure 2-5A). In agreement, in cell assay of sphingosine kinase activity further excluded the activation of SK after 2-AG treatment (Figure 2-5B). Indeed, the intracellular levels of S1P quantified by LC-MS/MS were not affected by the treatment with 2-AG (Figure 2-5B). Moreover, in order to further verify the involvement of SK in mediating 2-AG effect, SK1 and SK2 were efficaciously knocked-down by employing siRNA technology (Figure S 2-4B). As depicted in Figure 2-5C, the 2-AG-induced expression of COX2, IL-1β, IL-6 and IL-8 was unaffected by the downregulation of SK1 or SK2, thus demonstrating that the pro-inflammatory action of 2-AG did not depend on either SK1 or SK2 in endometriotic epithelial cells.

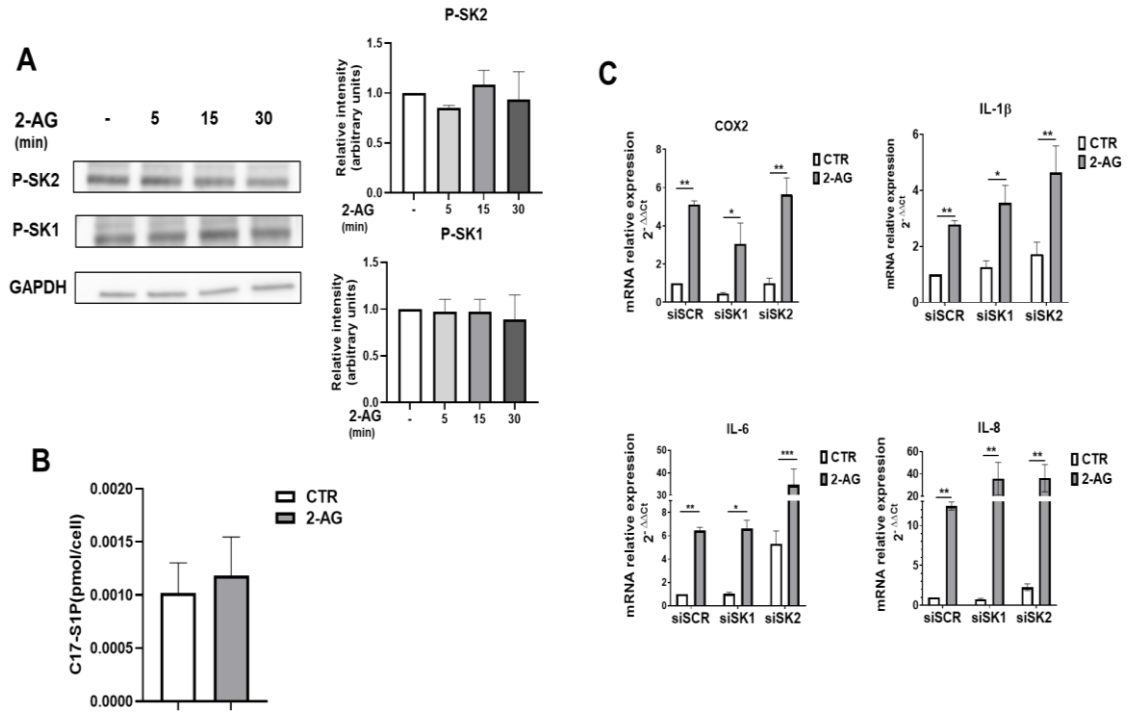

Figure 2-5. **2-AG pro-inflammatory action is SK-independent in endometriotic epithelial cells. (A)** Serum starved endometriotic epithelial cells were treated with 10 μM 2-AG for different time intervals (5, 15 and 30 min). Protein lysates were analyzed using SDS-PAGE electrophoresis and WB, using specific anti-phospho-SK1 (P-SK1), anti-phospho-SK2 (P-SK2) and anti-GAPDH antibodies. Histograms represent the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as band intensity normalized to the expression of GAPDH, fold change over control (set as 1). **(B)** Serum starved endometriotic epithelial cells were treated for 30 min with 10 μM 2-AG before being harvested and then subjected C17- S1P quantification by LC-MS/MS as described in the Material and Methods section. Results are the mean ± SEM of three independent experiments and are reported as pmol of S1P normalized on cell number. **(C)** Endometriotic epithelial cells transfected with SCR-, SK1- and SK2-siRNA were serum-starved prior to being challenged with 10 μM 2-AG for 24 h. mRNA quantitative analysis of COX2, IL-1β, IL-6 and IL-8 was performed by qPCR. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual inflammatory factors of the unchallenged specimen as reference gene.

2.2.6 Supplementary Figures

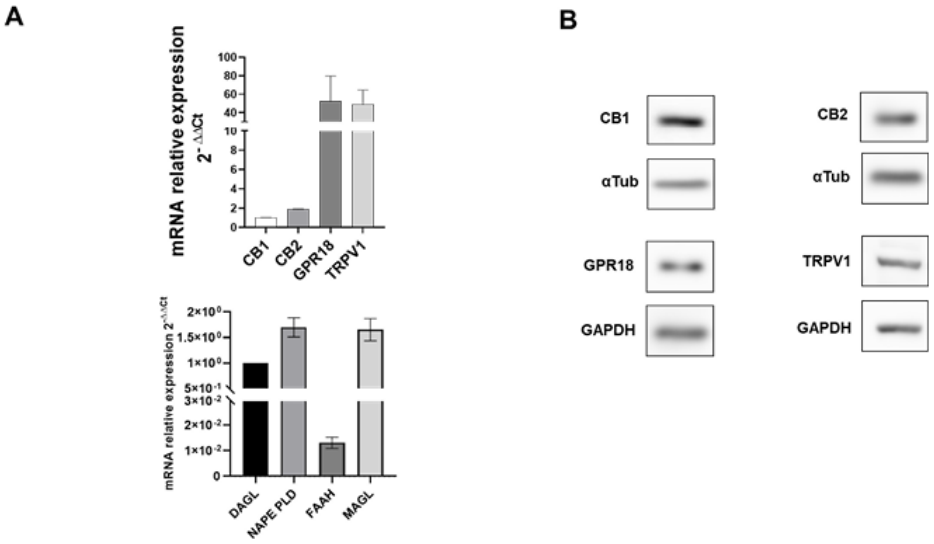

Figure S 2-1**. Endometriotic epithelial cells express cannabinoid receptors and enzymes. (A)** qPCR analysis was performed using TaqMan Gene Expression Assay probes specific for cannabinoid receptors CB1, CB2, GPR18 and TRPV1 as well as for the enzymes NAPE-PLD, DAGL, FAAH and MAGL in human endometriotic epithelial cells. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and CB1 (upper panel) or DAGL (lower panel) as reference gene. Data are mean ± SEM of three independent experiments. **(B)** WB analysis was performed using antibodies specific for CB1 (expected molecular weight ~53 kDa), CB2 (expected molecular weight ~40 kDa), GPR18 (expected molecular weight ~38 kDa) and TRPV1 (expected molecular weight ~95 kDa) in human endometriotic epithelial cells.

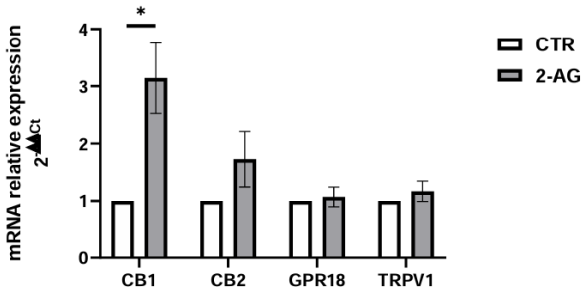

Figure S 2-2. **Effect of 2-AG on endocannabinoid receptor expression**. qPCR analysis was performed using TaqMan Gene Expression Assay probes specific for cannabinoid receptors CB1, CB2, GPR18 and TRPV1 in human endometriotic epithelial 12Z cells treated or not with 10 µM 2-AG for 24 h. Results, analyzed with $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and individual targets of the unchallenged specimen as reference gene. 2-AG increases CB1 mRNA levels in a statistically significant manner (Student's t-test $*p < 0.05$).

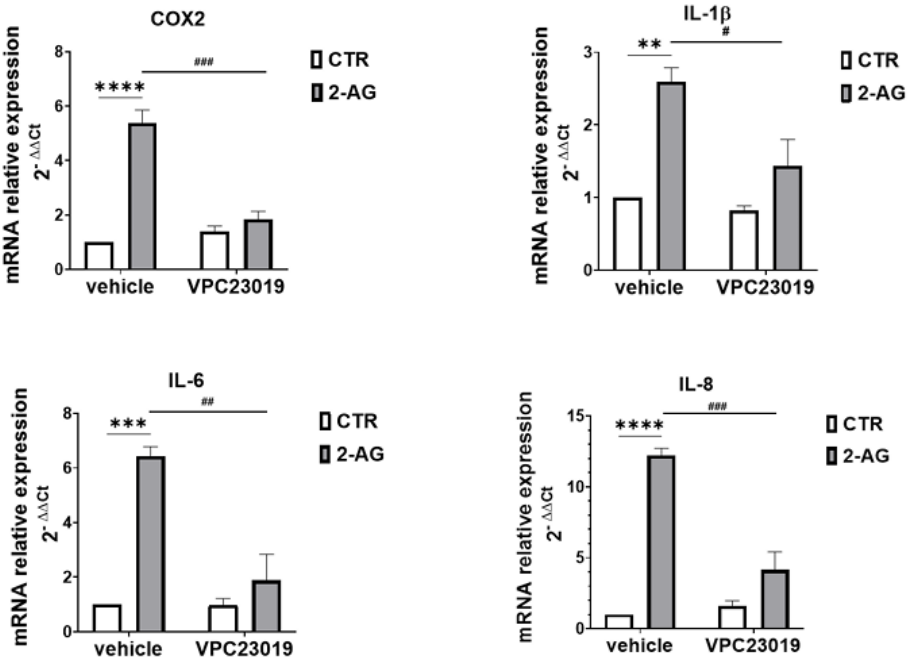

Figure S 2-3. **2-AG pro-inflammatory action relies on S1P3**. Serum-starved endometriotic epithelial cells were pretreated or not with the S1P1/S1P3 antagonist VPC23019 (10 μM) for 45min before being challenged with 10 μM 2-AG for 24 h. mRNA quantitative analysis of COX2, IL-1β, IL-6 and IL-8 was performed by qPCR. Results, analyzed with the 2-ΔΔCt method, were obtained using β-actin as housekeeping gene and individual inflammatory factors of the unchallenged specimen as reference gene. The effect of VPC23019 on 2-AG induced inflammatory effect was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

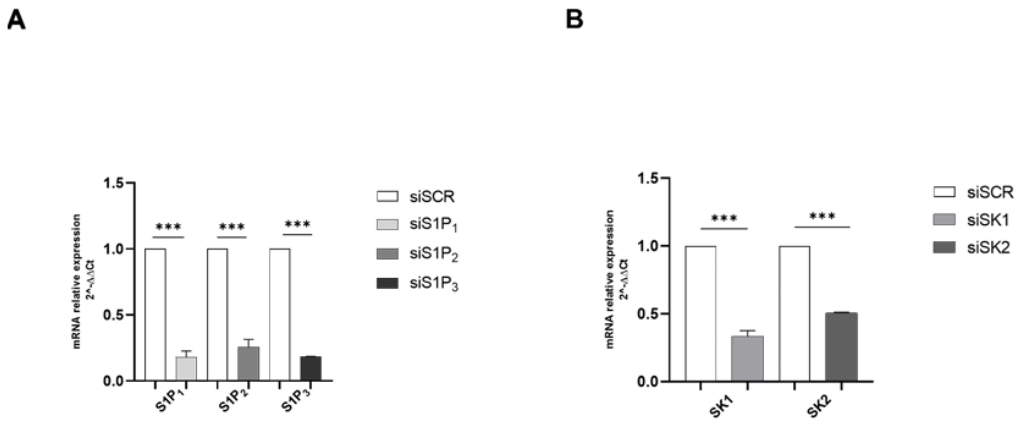

Figure S 2-4. **Silencing efficiency of S1PR and SKs in endometriotic epithelial cells**. **(A)** qPCR analysis was performed in endometriotic epithelial cells transfected with nonspecific siRNA (SCR) or with siRNA specific for $S1P_1$ or $S1P_2$ or $S1P_3$. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and each receptor subtype in cell transfected with siSCR used as calibrator. data are mean ± SEM of three independent experiments performed in triplicate. The effect of $S1P_1$- $S1P_2$- or $S1P_3$ -siRNA transfection is statistically significant by Student's t-test***$p < 0.001$. (B) qPCR analysis was performed in endometriotic epithelial cells transfected with nonspecific siRNA (SCR) or with siRNA specific for SK1 or SK2. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were obtained using β-actin as housekeeping gene and each receptor subtype in cell transfected with siSCR used as calibrator. data are mean ± SEM of three independent experiments performed in triplicate. The effect of SK1- or SK2-siRNA transfection is statistically significant by Student's t-test***$p < 0.001$.

In conclusion, our findings support the view that the specific $S1P_3$ isoform transduces the pro-inflammatory action of 2-AG in endometriotic epithelial cells and establish the rationale for the exploitation of $S1P_3$ targeting as an innovative non-hormonal approach to counteract endometriosis.

2.3 Discussion

Endometriosis is a chronic disease with life-impacting symptoms and high prevalence in women of reproductive age (Zondervan et al. 2020). Inflammation is a cardinal feature of endometriosis, strongly linked with its pathogenesis and the development of symptoms (Saunders and Horne 2021).

The cannabinoid receptors have long been known to play a role in inflammatory regulation through both pro-inflammatory and anti-inflammatory mechanisms, depending on the cellular environment and pathological state (Turcotte et al. 2016). Here, CB1, CB2, GPR18 and TRPV1 were found to be expressed in healthy endometrium and endometriotic lesions. The expression of CB1, CB2 and TRPV1 has been previously reported in the endometrium and endometriotic lesions (Almada et al. 2016; Leconte et al. 2010; Taylor et al. 2010; Allam et al. 2022; Lingegowda et al. 2022), while this study represents the first experimental evidence as regards the expression of the receptor GPR18 in endometriosis. Our findings are in accordance with a previous work by Sanchez and coworkers (A. M. Sanchez et al. 2016) showing that CB1, CB2 and TRPV1 transcript levels were not different between endometrial stromal cells from endometriosis-affected women and healthy controls in the proliferative phase. Conversely, a significant increase in TRPV1 mRNA levels was observed in rectosigmoid deep infiltrating endometriosis nodules (Bohonyi et al. 2017). Based on immunohistochemical analyses, endocannabinoid receptors CB1 and CB2 were found to be downregulated in endometriotic tissues (Bilgic et al. 2017), while TRPV1 protein levels were increased in rectosigmoid deep infiltrating endometriosis nodules (Bohonyi et al. 2017). Analogously, in accordance with the literature (A. M. Sanchez et al. 2016; Lingegowda et al. 2022; Bilgic et al. 2017) we here demonstrate the expression of the endocannabinoid metabolic enzymes NAPE-PLD, DAGL, FAAH and MAGL in healthy endometrium and endometriotic lesions. Limited literature data are available as regards the expression of endocannabinoid-metabolizing enzymes in endometriotic lesions. In particular, it was shown that FAAH and NAPE-PLD transcripts did not significantly differ between endometrial stromal cells from endometriosis-affected women and healthy controls (A. M. Sanchez et al. 2016) whereas NAPE-PLD, DAGL, FAAH and MAGL protein levels were reduced in endometriotic lesions (Bilgic et al. 2017). It will be interesting to analyze in future studies whether the expression of ECS components varies across endometriotic lesions of different anatomical localizations, with the aim of correlating the dysregulation of ECS system to disease phenotype and severity.

In this study 2-AG, but not the non hydrolyzable anandamide analogue, MAEA, was found to strongly upregulate mRNA levels of COX2, enzyme responsible for the conversion of arachidonic acid into prostanoids including prostaglandins, prostacyclin, and thromboxane, all lipid mediators critically involved in the regulation of inflammation and pain perception (Simon 1999). Moreover, 2-AG augmented the release of pro-inflammatory cytokines IL-1β, IL-6 and IL-8 in endometriotic epithelial cells, confirming the role of ECS in the modulation of inflammation. Differential biological roles of endocannabinoid species have been previously extensively reported. For instance, 2-AG, but not AEA, exerted neuroprotective effects on granule cells in the dentate gyrus of the hippocampus (Kreutz et al. 2007), while only AEA inhibited amyloid β aggregation (Khavandi et al. 2023).

In accordance with the here reported proinflammatory role of 2-AG in endometriotic cells, increased levels of the endocannabinoid in the peritoneal fluid of women affected by endometriosis positively correlated with PGE2 concentration, key mediator of inflammation (Andrieu et al. 2022). Moreover, in endometrial inflammation the selective activation of CB2 was demonstrated to be associated with nitric oxide release, a key player in immune regulation and inflammation (Iuvone et al. 2008). 2-AG has also been reported to increase the production of chemokines in HL-60 cells (Sugiura et al. 2006), while its seminal plasma levels are higher in the presence of inflammation (Barbonetti et al. 2017). However, in animal models of endometriosis, treatment with the two main constituents of the plant *Cannabis sativa* able to bind the cannabinoid receptors, D9-tetrahydrocannabinol and cannabidiol, was shown to ameliorate the endometriosis-associated pain (Escudero-Lara et al. 2020) and inflammation (Okten et al. 2023), respectively, pointing at differential effects exerted by phytocannabinoids and endocannabinoids. It is worth noting that both AEA and 2-AG exert anti-inflammatory effects reducing the production of pro-inflammatory cytokines in immune cells (Berdyshev et al. 1997; Chang et al. 2001; Cencioni et al. 2010). In addition, 2-AG decreased the expression of COX2 elicited by different inflammatory stimuli in hippocampal neurons (Zhang and Chen 2008; Chen et al. 2011) and leptin-induced ROS formation in hypothalamic neurons (Palomba et al. 2015).

Here, an interplay between ECS and S1P signaling in the production of inflammatory factors has been revealed in endometriosis. Indeed, 2-AG was found to modulate S1P signaling axis since the treatment with the endocannabinoid upregulated the mRNA and protein content of $S1P_3$, while it augmented SK1 and Spns2 only at transcriptional level. The here reported findings showing S1P signaling modulation by ECS are in line with a previous study by

Standoli et al. (Standoli et al. 2023) in which it was demonstrated that pharmacological stimulation of CB2 counteracted the LPS-induced increase of SK1 and SK2 transcription in microglia cells. Reciprocally, ECS signaling regulation by S1P has been shown since the sphingolipid augmented the expression of TRPV1, while it reduced CB2 levels in skeletal muscle cells (Standoli et al. 2022). Interestingly, $S1P_3$ was here demonstrated to mediate the pro-inflammatory action of 2-AG since its pharmacological inhibition or down-regulation by gene silencing significantly reduced the elevated expression of COX2, IL-1β, IL-6 and IL-8 elicited by the endocannabinoid. These results provide compelling evidence that the upregulation of the analyzed pro-inflammatory molecules by 2-AG relies on $S1P_3$ engagement. However, this does not exclude that, in a reciprocal manner, the 2-AG–induced upregulation of $S1P_3$ is at least in part mediated by pro-inflammatory factors, thereby supporting a positive feedback mechanism that sustains inflammation. Notably, our research group has already provided multiple evidence that $S1P_3$ is crucially involved in endometriosis pathogenesis. Indeed, this receptor subtype has been found to be upregulated in endometriotic lesions (Bernacchioni et al. 2024; Bernacchioni, Capezzuoli, et al. 2021) and to positively correlate with the fibrosis extent of the disease (Bernacchioni et al. 2024). Moreover, $S1P_3$ mediated the pro-fibrotic action of S1P (Bernacchioni et al. 2024) as well as the pro-invasive phenotype elicited by neuropeptide S (Prisinzano et al. 2024) in endometriotic epithelial cells. Notably, in relation to the regulation of IL-8 levels, the data presented in this study demonstrated not only the involvement of $S1P_3$, similarly to what was observed for the other cytokines, but also a further positive role for $S1P_1$ and a negative regulatory role for $S1P_2$. Indeed, when this latter receptor isoform was silenced in endometriotic epithelial cells, IL-8 levels significantly increased following treatment with 2-AG, compared to control cells. This role of $S1P_2$ contrasts with previous findings in extravillous trophoblast-derived HTR-8/SVneo cells, where IL-8 release was dependent on the $S1P_2$/Rho signaling axis, highlighting that such regulatory mechanisms are highly context-specific (Brünnert et al. 2015).

Compelling evidence from the literature indicates that various extracellular cues elicit their biological effects through the regulation of SK1 and SK2, causing enhanced production of S1P, that after its export outside the cell can engage its cognate receptors (S1PR) (Spiegel and Milstien 2011). Crucially here, 2-AG was demonstrated to be unable to enhance the catalytic activity of SK and consistently, SK specific downregulation did not interfere with its pro-inflammatory action, thus ruling out the involvement of SK/$S1P_3$ inside-out signaling in endometriotic epithelial cells. On the contrary, SK1 activation induced by the endocannabinoid

anandamide was required to recruit $S1P_3$ and mediate vasorelaxation in rat coronary artery (Mair et al. 2010) and SK1/S1P regulatory-axis was necessary for the rapid hypotension induced by anandamide in anaesthetised mouse (Greig et al. 2019).

The action of 2-AG likely depends on its interaction with one or more specific receptors, found to be expressed in the endometriotic epithelial cells. However, our preliminary experimental approaches aimed at identifying the cannabinoid receptor involved in mediating the pro-inflammatory effect of 2-AG, so far have not yielded robust results. Since the data presented here show that SK1/SK2 are not required to mediate the effects of 2-AG, one possible explanation for the activation of $S1P_3$ by 2-AG is a direct interaction between cannabinoid and $S1P_3$ receptors. Further studies are required to clarify the molecular mechanism by which 2-AG leads to $S1P_3$ transactivation in endometriotic cells. Of note, S1PR and CB1/CB2 belong to class A of GPCR and share 20% sequence identity. Notably, $S1P_5$ has been reported to exert a negative regulation of the tumorigenic effect induced by CB2 in glioblastoma cells: bioluminescence resonance energy transfer analysis highlighted that this S1P receptor subtype strongly and specifically interacts with CB2 (Kim and Ghil 2025).

Collectively, these findings enhance our understanding of the molecular mechanisms involved in the development of endometriosis pathogenesis, highlighting the critical role of $S1P_3$ in mediating the pro-inflammatory action of 2-AG in endometriotic epithelial cells. S1PR modulators have emerged as promising therapeutics for various immune-mediated diseases, including multiple sclerosis, inflammatory bowel disease and psoriasis, by modulating lymphocyte trafficking and reducing tissue inflammation (McGinley and Cohen 2021). Since we recently demonstrated that $S1P_3$ is involved in the fibrotic and invasive trait of endometriotic cells (Prisinzano et al. 2024; Bernacchioni et al. 2024), the here presented data further support the potential of targeting $S1P_3$ as a novel non-hormonal therapeutic strategy for endometriosis treatment.

3 . Paper 2

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Expression of cannabinoid receptors CB1, CB2 and GPR18 in adenomyotic lesions

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3.1 Methods

3.1.1 Patients and tissue collection

The here presented study was conducted on histologically-diagnosed, formalin-fixed paraffin-embedded (FFPE) samples of non-pregnant patients during the proliferative phase undergoing hysterectomy at Careggi University Hospital in Florence, Italy. Women affected by adenomyosis stopped all hormonal treatments at least three months before surgery. Control endometrial specimens were collected during diagnostic hysteroscopy from nonpregnant healthy women not affected by any uterine disorder, during the proliferative phase. The clinical and imaging investigations excluded endometriosis and other uterine disorders. All samples were histologically characterized. All the enrolled women provided written informed consent, and their privacy rights were observed. The study protocol was approved by the Institutional Review Board (number 13742).

3.1.2 Quantitative real time PCR (qPCR)

Total RNA was extracted from FFPE tissue samples using the All-Prep DNA/RNA FFPE kit (Quiagen) and reverse transcribed (300 ng) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific), as suggested by the manufacturer and following the instruction at section 2.1.5.

3.1.3 Immunohistochemistry (IHC)

Tissue samples were fixed with formalin and paraffin embedded. Serial 3 μm sections were obtained from each block, and subsequent slides were stained for IHC analysis of the cannabinoid receptors CB1, CB2 and GPR18. The IHC was performed as it is described in section 2.1.6. The slices were visualized with an optical microscope, and the images were obtained using a Evos xl core imaging system, 10× magnification.

3.1.4 Statistical analysis

Shapiro-Wilk normality test and Mann-Whitney test were used for statistical analysis; $p<0.05$ was statistically significant. Graphical representations were realized using GraphPad Prism 8.3 (GraphPad Software).

3.2 Results

3.2.1 Cannabinoid receptors expression in Adenomyosis

The mRNA expression of the cannabinoid receptors CB1, CB2 and GPR18 was evaluated in adenomyotic tissues (n = 22) compared to healthy endometrial specimens (n = 16). As depicted in Figure 3-1A, the expression of all the analyzed cannabinoid receptors resulted to be significantly higher in adenomyotic samples ($P < 0.0001$) (Figure 3-1A). Notably, IHC analysis of CB1, CB2 and GPR18 revealed a positive staining in the glandular and stromal components both in the adenomyotic lesions as well as in the control healthy endometrium (Figure 3-1B). The protein expression levels of CB1, CB2 and GPR18 in adenomyotic lesions were not significantly different from those observed in healthy endometrium (Figure 3-1B). However,

it should be taken into consideration that this experimental approach, aimed to confirm the findings on the cannabinoid receptors expressed in adenomyotic lesions obtained at mRNA level, is less quantitative than qPCR.

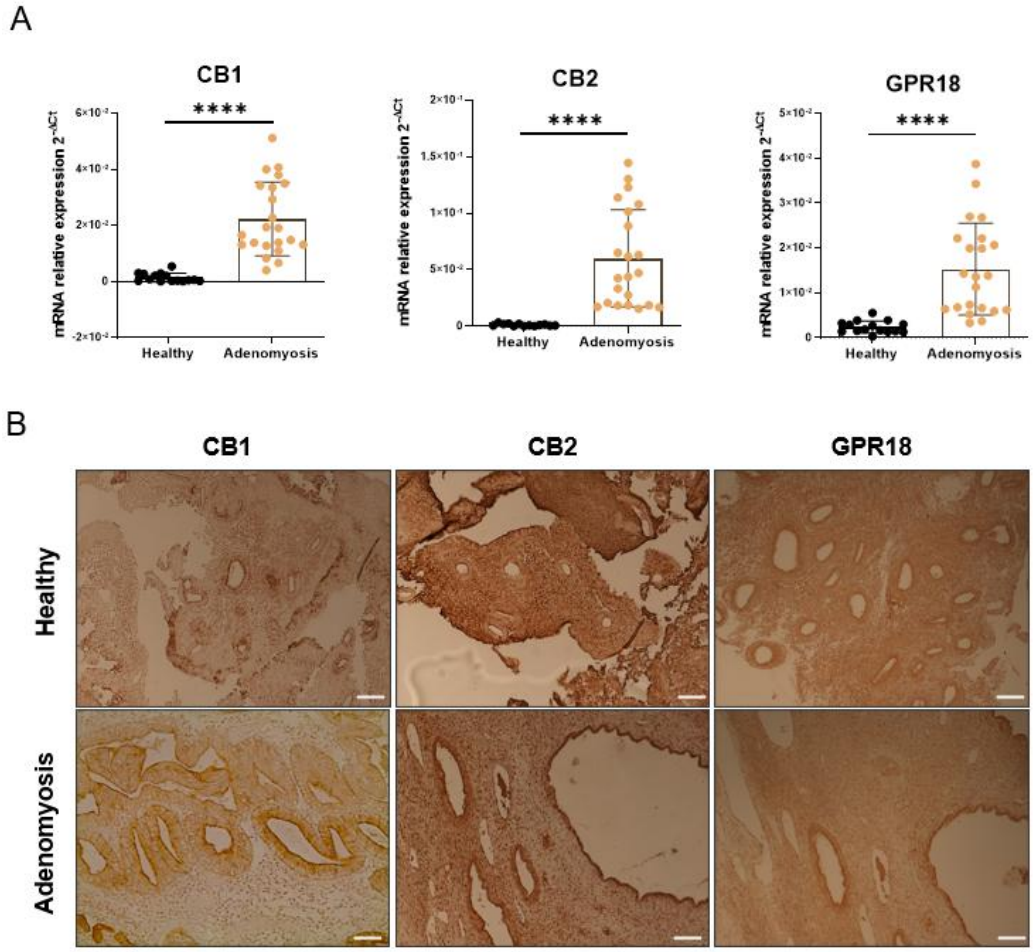

Figure 3-1. **Cannabinoid receptors expression in Adenomyosis**. Cannabinoid receptors are expressed in endometrial and adenomyotic tissues. **(A)** Real-time polymerase chain reaction analysis was performed in total RNA extracted from healthy endometrium (Healthy, n=16) and nodular adenomyotic tissue (Adenomyosis, n=22). Results were normalized to ACTB housekeeping gene and analyzed with the $2^{-\Delta Ct}$ method. The different expression of CB1, CB2 and GPR18 are statistically significant according to Mann-Whitney (****P<0.0001). **(B)** IHC analysis of CB1, CB2 andGPR18 in adenomyotic lesions from patients affected by adenomyosis (Ademomyosis, n=9) and endometrium from women without any uterine disorder (Healthy, n=5). Magnification: 10×, scale bar: 150 μm.

3.3 Discussion

In this study, we provide evidence that the mRNA levels of CB1, CB2 and GPR18 are augmented in adenomyotic tissue in comparison to healthy endometrium. Our findings are in accordance with a previous work by Shen and coworkers (2019) showing that CB1 and CB2 mRNA and protein expression levels were augmented in the junctional zone and outer myometrium of women affected by adenomyosis (Shen, Duan, Wang, Hong, et al. 2019). In addition, the higher expression of CB1 in the junctional zone of adenomyosis patients was shown to positively correlate with proliferating cell nuclear antigen, underlying an alteration of cell proliferation in this uterus compartment of adenomyosis patients (Wang et al. 2021). Conversely, the same authors reported that CB1 and CB2 expression levels are significantly reduced in eutopic and ectopic endometrium of women affected by adenomyosis in comparison to endometrium of healthy controls (Shen, Duan, Wang, Gan, et al. 2019). Moreover, in the endometrium of women affected by adenomyosis and endometriosis a lower immune labelling of CB1 and CB2 was described compared to healthy women (Bilgic et al. 2017). The discrepant findings described above may be due to a limited sample size and to differences in the experimental setting between the various studies.

Moreover, this study is the first experimental evidence regarding the expression of the receptor GPR18 in adenomyotic lesions. Interestingly, GPR18 was previously shown to mediate the pro-migratory action of different endocannabinoid agonists in endometrial cells (Gentilini et al. 2010; McHugh et al. 2012), suggesting that the receptor might be involved in mediating the invasiveness of endometriotic cells into the myometrium and in the development of adenomyotic lesions. Currently, adenomyosis is considered an estrogen-dependent disease characterized by augmented inflammation and fibrosis (Vannuccini et al. 2024; 2017). Solid evidence establishes a key role for cannabinoid receptors in the inflammatory process and in immune cell regulation (Leuti et al. 2020). On these bases, altered expression of cannabinoid receptors at the level of adenomyosis lesions might be involved in the pathogenesis of adenomyosis influencing inflammatory changes and fibrogenesis. Therefore, cannabinoid receptor targeting could be considered to identify innovative molecular therapeutic targets for the disease.

4 . Paper 3

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Sphingosine1-phosphate acts as proliferative and fibrotic cue in leiomyoma cells

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4.1 Material and Methods

4.1.1 Cell culture and treatments

The myometrial (A009) and leiomyoma (A010) cell lines, immortalized with human papilloma virus type 16 were provided by Dr. William H Catherino, MD, PhD (Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland) (Malik et al. 2008). The cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM): Nutrient Mixture F-12 Ham (F12) (Merck Life Sciences, Burlington, MA, USA) supplemented with 10% fetal bovine serum (Merck Life Sciences, Burlington, MA, USA), 1% penicillin-streptomycin (Merck Life Sciences, Burlington, MA, USA), 1% fungizone (amphotericin B) (Euroclone, Milan, Italy) and 1% glutamine (Merck Life Sciences, Burlington, MA, USA) at 37° C in 95% air 5% CO_2. The cells were starved overnight prior to every treatment in the starvation medium (DMEM:F12 supplemented with 10% charcoal stripped fetal bovine serum (Merck Life Sciences, Burlington, MA, USA), 1% penicillin-streptomycin, 1% amphotericin B and 1% glutamine). When required, cells were preincubated with pharmacological inhibitors or antagonists (ezrin inhibitor, NSC668394; the ERK1/2

inhibitor, U0126; the $S1P_1/S1P_3$ antagonist, VPC23019 and the $S1P_2$ antagonist, JTE013 from Merck Life Sciences, Burlington, MA, USA) 1 h before the treatment with S1P (Merck Life Sciences, Burlington, MA, USA). Institutional review board protocol no. 13742.

4.1.2 Bromodeoxyuridine (BrdU) Cell Proliferation Assay

Myometrial and leiomyoma cells were seeded in 96-well plates. Cell proliferation was evaluated using the Bromodeoxyuridine (BrdU) Cell Proliferation Assay (Merck Life Sciences, Burlington, MA, USA) according to manufacturer's instructions. Briefly, the cells were incubated with BrdU for the last 24 h of treatment, fixed, incubated with the anti-BrdU antibody and with the secondary HRP conjugated antibody. Finally, substrate was added and the absorbance read using a spectrophotometric plate reader (Tecan Trading AG, Switzerland).

4.1.3 Western Blot (WB) Analysis

The protein lysates were prepared, and WB analysis was performed as described in section 2.1.7. The membranes were incubated with primary antibodies against the targets of interest (anti-phospho-ERM and anti-phospho-ERK1/2 (Cell Signaling Technology, Danvers, Massachusetts, USA); anti-N-cadherin, anti-vinculin and anti- Collagen type I alpha 1 (COL1A1)(Santa Cruz Biotechnology, Santa Cruz, CA); anti-αSMA) (Merck Life Sciences, Burlington, MA, USA); anti-transgelin (Everest Biotech Ltd, Upper Heyford, UK); anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Densitometric analysis was performed by the ImageJ software (National Institutes of Health, USA). Band intensity of target proteins was reported as fold increase relative to the respective control, set as 1.

4.1.4 Statistical Analysis

Data were analyzed as described at section 2.1.10

4.2 Results

4.2.1 S1P stimulates leiomyoma but not myometrial proliferation via $S1P_2$

The effect of S1P on the induction of proliferation was evaluated in myometrial and leiomyoma cells using the BrdU incorporation assay. Obtained results (Figure 4-1) show that 48 h stimulation with 1 μM S1P significantly increased the proliferation of leiomyoma cells, but not that of myometrial cells. In order to characterize the S1PR implicated in S1P-induced proliferation, leiomyoma cells were pretreated with selective $S1P_1$ antagonist W146 (10 μM), $S1P_2$ antagonist JTE013 (1 μM), or $S1P_{1/3}$ antagonist VPC23019 (10 μM) or $S1P_4$ antagonist CYM50358 (1 μM). Data reported in Figure 4-2 clearly shows that the mitogenic effect elicited by S1P was abolished in the presence of JTE013, demonstrating that the sphingolipid stimulates cellular proliferation via $S1P_2$ signaling in leiomyoma cells. In contrast, the pharmacological blockade of the other receptor isoforms did not affect the mitogenic effect elicited by S1P in leiomyoma cells (Figure 4-2).

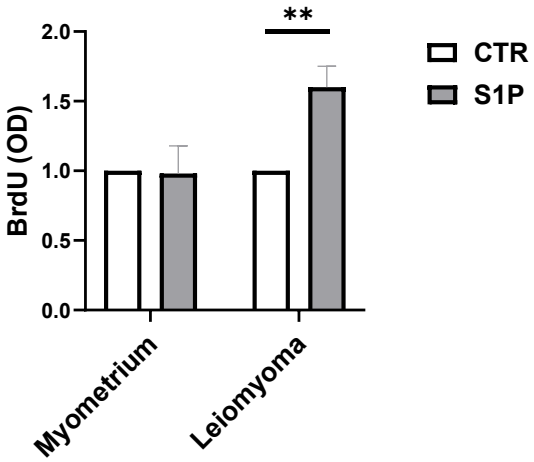


Figure 4-1**. S1P stimulates proliferation in leiomyoma but not myometrial cells.** Human myometrial or leiomyoma cells were treated for 48 h with 1 μM S1P and cell proliferation was evaluated with the BrdU incorporation assay. Data are shown as the mean ± SD of three independent experiments. The BrdU incorporation of the treated cells is reported as relative to the control, set as 1. Differences are statistically significant according to Student's t-test (**P<0.01).

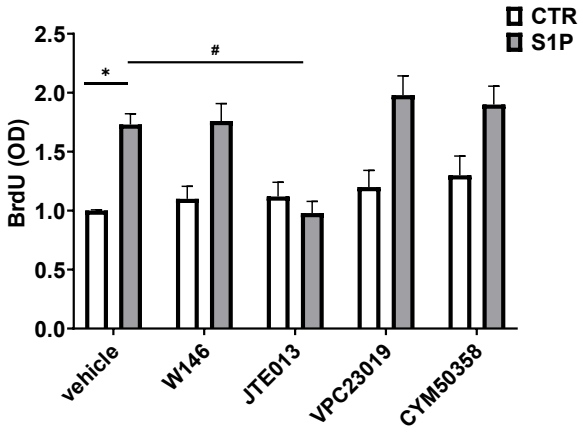


Figure 4-2. **S1P-stimulated proliferation in leiomyoma cells is dependent on $S1P_2$ engagement.** Human leiomyoma cells were pre-treated for 1 h with the specific $S1P_1$ antagonist W146 (10 μM), $S1P_2$ antagonist JTE013 (1 μM), $S1P_1$/$S1P_3$ antagonist VPC23019 (10 μM), or $S1P_4$ antagonist CYM50358 (1 μM), before being challenged for 48 h with 1 μM S1P. Cell proliferation was evaluated with the BrdU incorporation assay. Data are shown as the mean ± SD of three independent experiments. The BrdU incorporation of the cells is reported as relative to the untreated control, set as 1. The effect of $S1P_2$ blockade in S1P-induced proliferation (*$P < 0.05$) was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (#$P < 0.05$).

4.2.2 S1P-induced leiomyoma cell proliferation requires ERK1/2 and ERM activation

The possible involvement of ERK1/2 and ERM activation in the S1P mitogenic effect was then investigated. WB analysis of phosphorylated ERM (p-ERM) and ERK1/2 (p-ERK1/2) performed in leiomyoma cells incubated with 1 μM S1P for different time intervals (1, 5, 10, 15, 30 min) revealed that the sphingolipid significantly activated both ERM and ERK1/2. In particular, ERM phosphorylation was maximal between 1 and 10 min and still high at 30 min, while ERK1/2 were maximally activated between 5 and 10 min (Figure 4-3A). Next, in order to evaluate the involvement of ERK1/2 and ERM activation in the S1P-induced leiomyoma proliferation, specific pharmacological inhibitors for ERK1/2 (U0126, 5 μM) or ezrin (NSC668394,1 μM) were used to pre-treat the cells prior to stimulation with 1 μM S1P for 48 h. As shown in Figure 4-3B, the inhibition of ERK1/2 and ezrin abolished the proliferative effect of S1P, demonstrating a crucial role of both signaling pathways in mediating the mitogenic effect of the sphingolipid.

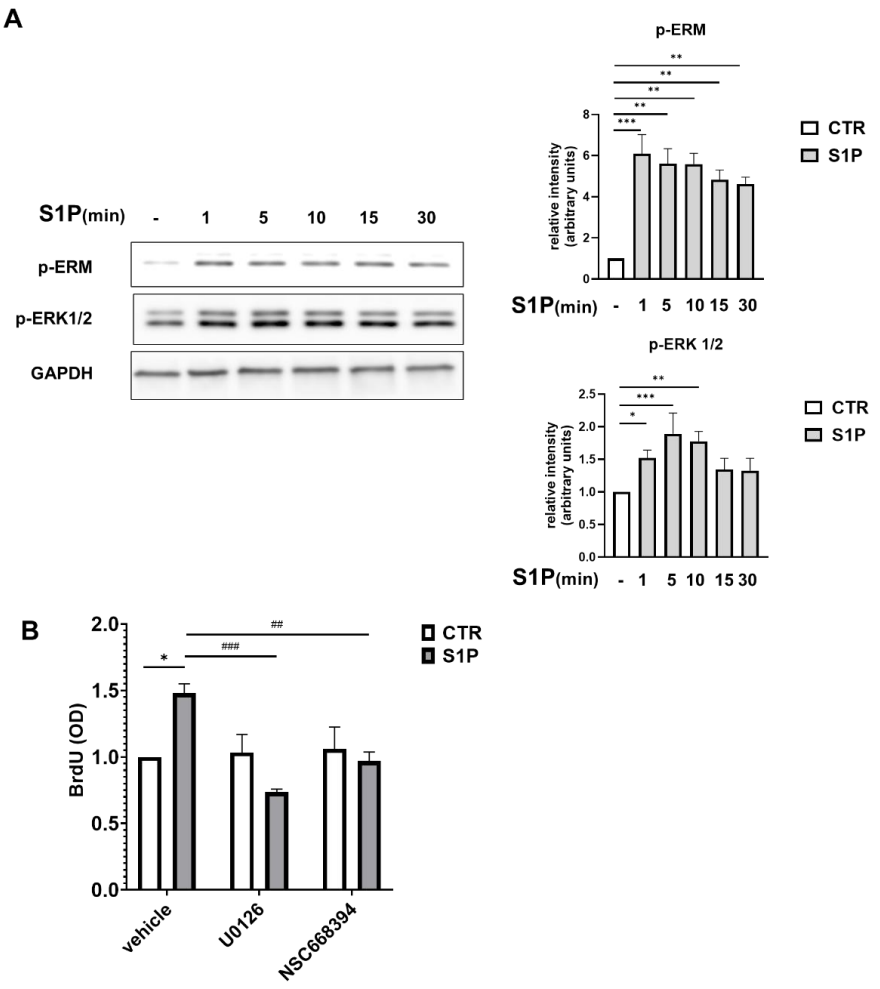
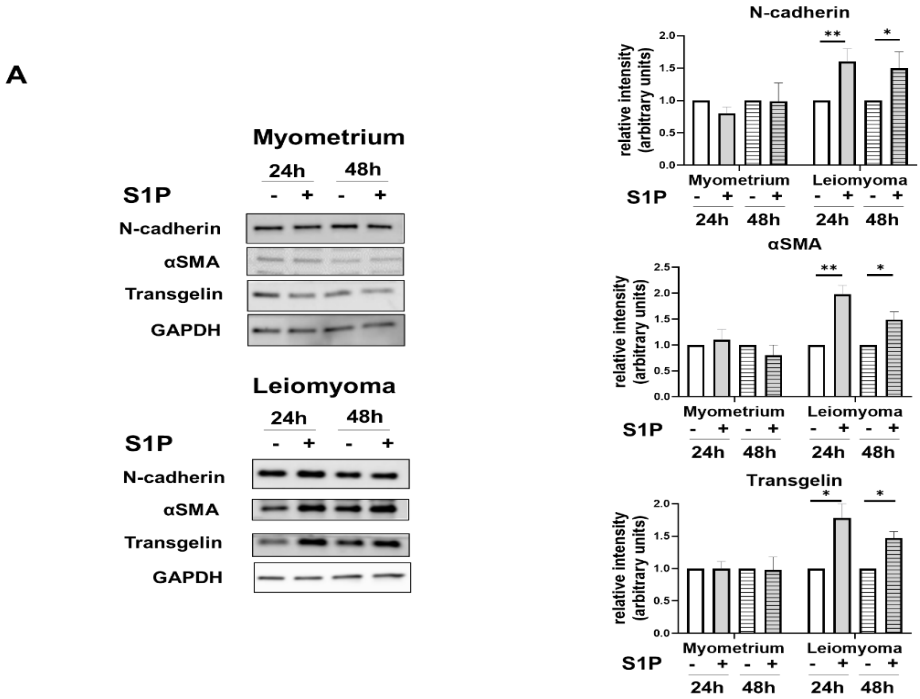


Figure 4-3. **S1P stimulates leiomyoma cell proliferation via ERK1/2 and ezrin. (A)** WB analysis was performed in leiomyoma cells following treatment with 1 μM S1P for the indicated time points, using specific antibodies against p-ERM and p-ERK1/2. A blot representative of three independent experiments with analogous results is shown. The histograms represent the mean ± SD of the densitometric analysis of three independent experiments. Data are reported as protein expression normalized to GAPDH, fold change over control, set as 1. Differences are statistically significant according to one-way ANOVA followed by Bonferroni post hoc test (*P<0.05, **P<0.01, ***P<0.001). **(B)** Leiomyoma cells were pre-treated for 1 h with the specific ERK1/2 inhibitor U0126 (5 μM) or the ezrin inhibitor NSC668394 (1 μM) before being challenged for 48 h with 1 μM S1P. Cell proliferation was evaluated with the BrdU incorporation assay. Data are shown as the mean ± SD of three independent experiments. The BrdU incorporation of the cells is reported as relative to the untreated control, set as 1. The effect of ERK1/2 or ezrin inhibition in S1P-induced proliferation (*P < 0.05) was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (##P < 0.01; ###P < 0.001).

4.2.3 S1P induces fibrosis in leiomyoma but not myometrial cells

WB analysis of fibrotic markers (N-cadherin, αSMA and transgelin) was performed both in leiomyoma and myometrial cells stimulated with 1 μM S1P for 24 and 48 h. Data shown in Figure 4-4A demonstrated that S1P exerted a pro-fibrotic action in leiomyoma cells, significantly increasing the expression of the evaluated fibrotic markers at both time points of treatment. In contrast, S1P did not affect the levels of the same fibrotic markers in myometrial cells (Figure 4-4A). S1P pro-fibrotic effect in leiomyoma cells was mediated by S1PR. Indeed, the increase in the expression of N-cadherin, αSMA, transgelin and COL1A1 elicited by the bioactive sphingolipid was abolished by the pre-treatment with the pharmacological antagonists of $S1P_1$/$S1P_3$ (VPC23019, 10 μM) and $S1P_2$ (JTE013, 1 μM) (Figure 4-4B).



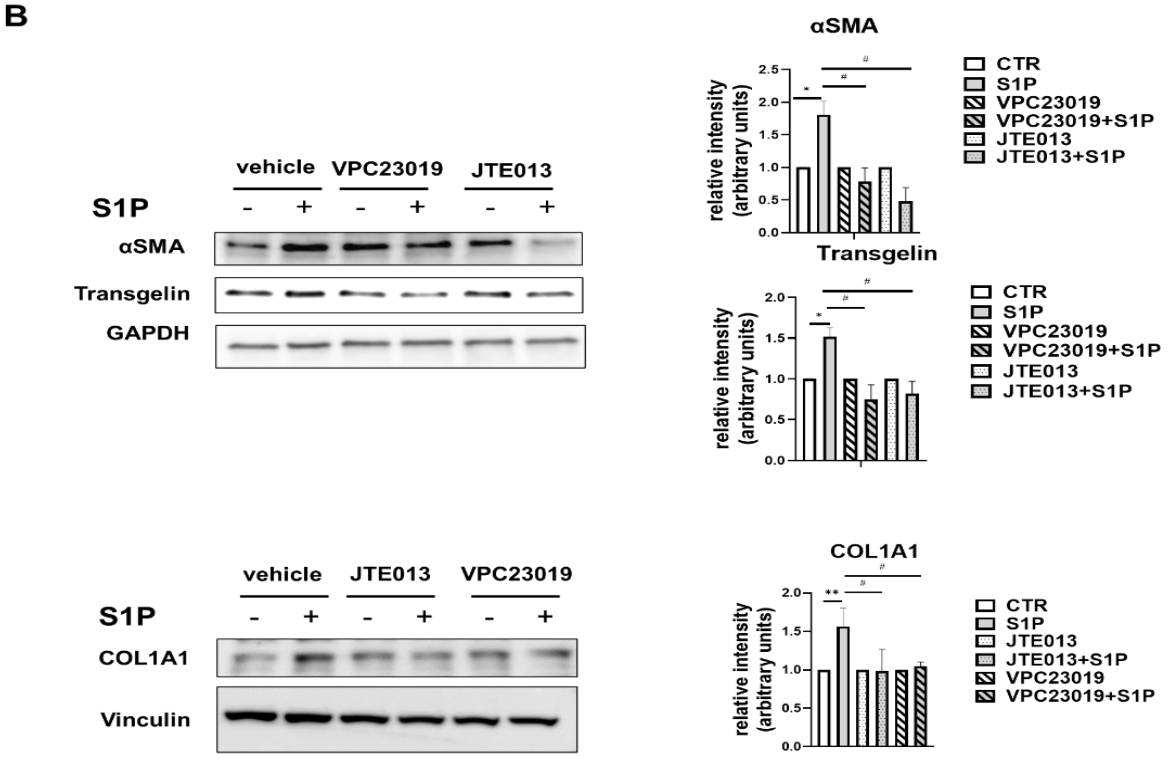


Figure 4-4. **S1P induces fibrosis in leiomyoma but not myometrial cells. (A)** WB analysis was performed in myometrial or leiomyoma cells following treatment with 1 μM S1P for 24 h or 48 h, using specific antibodies against N-cadherin, αSMA and transgelin. Representative blots of three independent experiments with analogous results are shown. The histograms represent the mean ± SD of the densitometric analysis of three independent experiments. Data are reported as protein expression normalized to GAPDH, fold change over control, set as 1. Differences are statistically significant according to one-way ANOVA followed by Bonferroni post hoc test (*P<0.05, **P<0.01). **(B)** Leiomyoma cells were pre-treated for 1 h with the $S1P_1$/$S1P_3$ antagonist VPC23019 (10 μM) or the $S1P_2$ antagonist JTE013 (1 μM) before being challenged with 1 μM S1P for 24 h. WB analysis was performed using specific antibodies against αSMA, transgelin and COL1A1. A blot representative of three independent experiments with analogous results is shown. The histograms represent the mean ± SD of the densitometric analysis of three independent experiments. Data are reported as protein expression normalized to GAPDH or vinculin, fold change over control, set as 1. The effect of $S1P_1$/$S1P_3$ or $S1P_2$ blockade in S1P-induced fibrosis (*P < 0.05) was statistically significant by two-way ANOVA followed by Bonferroni's post hoc test (#P < 0.05).

4.3 Discussion

Despite the high prevalence of uterine leiomyomas, the molecular mechanisms underlying the pathogenesis and development of the disease are yet to be fully clarified (Islam et al. 2013; Walker and Stewart 2005; Ramaiyer et al. 2024; Ali et al. 2023; Yang et al. 2022; Stewart et al. 2016; Ono et al. 2013).

In this study, the biological action of the bioactive sphingolipid S1P in leiomyoma cells has been deeply investigated. The obtained results clearly demonstrate that S1P exerts a crucial role in leiomyoma, promoting fundamental processes for its pathogenesis, such as proliferation and fibrosis. S1P signaling pathway has been recently shown to be deeply dysregulated in uterine fibroids: the expression levels of the enzymes SK1 and SK2 and of the receptors $S1P_2$, $S1P_3$ and $S1P_5$ are significantly increased in leiomyoma tissue compared to the adjacent myometrium (Bernacchioni, Ciarmela, et al. 2021).

Here, S1P has been shown to potently stimulate BrdU incorporation into DNA in leyomioma cells but not in myometrial cells. Accordingly, the role of S1P in regulating cell proliferation is well established both in physiological (Olivera and Spiegel 1993; Calise et al. 2012) and pathological conditions, being implicated in the progression of different types of cancer (Hirose et al. 2018; Miura et al. 2021; Nagahashi and Miyoshi 2024; Nojima et al. 2024). To investigate the receptor isoform specifically involved in the S1P-induced mitogenic effect, S1PR antagonists were employed. Interestingly, $S1P_2$ was found to be implicated in the transmission of S1P-induced cell proliferation, similarly to what previously demonstrated in mesoangioblasts (Donati et al. 2007) and satellite cells (Calise et al. 2012). Considering that increased proliferation is one of the first pathogenetic mechanism of uterine fibroids (Islam et al. 2013; Ono et al. 2013), $S1P_2$ could be proposed as a potential target for their treatment.

In this study, the molecular mechanisms by which S1P stimulates leiomyoma cell proliferation were also investigated. S1P rapidly and potently increased the activation of ERK1/2 and ERM in leiomyoma cells. Both ERM and ERK1/2 signaling pathways were found to be necessary for the mitogenic response to S1P, since the selective pharmacological inhibition of ERK1/2 and ezrin abrogated the biological action of the sphingolipid. The activation of ERK1/2 has long been associated with mitogenic signaling being also aberrantly activated in different types of cancer (Chen et al. 2019; Niu and Ji 2022; Omokehinde et al. 2020). Notably, ERK1/2 signaling transduces the proliferative effect of 17β-estradiol (Nierth-Simpson et al. 2009) and leptin (Reschke et al. 2022) in leiomyoma cells. Data reported here

are in agreement with a number of other studies that established a crucial role for ERK1/2 activation in the mitogenic effect of S1P (Donati et al. 2007; Kimura et al. 2000; Van Brocklyn et al. 2002; Pébay et al. 2001).

The S1P-dependent activation of the ERM complex described in the present study is in agreement with previous findings on epithelialotic vesicle progenitors (Cencetti et al. 2019), breast cancer (Canals et al. 2010), vascular smooth muscle (Morris et al. 2018) and epithelial endometriotic (Bernacchioni et al. 2024) cells. Besides being implicated in cellular migration and invasion (Yanan Song et al. 2020), in accordance with our findings, it has been reported that ERM proteins modulate cellular proliferation in rheumatoid fibroblast-like synoviocytes (Huang et al. 2011) and ovarian cancer cells (M. J. Li et al. 2021).

Beyond identifying S1P as key regulator of cell proliferation, data reported here demonstrate a crucial role of the sphingolipid, via ligation to its receptors, in the promotion of fibrosis in leiomyoma cells. Indeed, S1P significantly increased the levels of the fibrotic markers N-cadherin, αSMA, transgelin and COL1A1 in leiomyoma cells while it did not alter their levels in myometrial cells. These data corroborate our previous findings obtained at mRNA level (Bernacchioni, Ciarmela, et al. 2021). The S1P signaling pathway has been crucially linked to the development of fibrosis in different tissues, such as skeletal muscle, lung, kidney and heart (Donati et al. 2021). Of note, we have recently demonstrated that SK/S1P signaling axis, through the specific engagement of $S1P_2$/$S1P_3$, mediates the pro-fibrotic action of activin A in leiomyoma cells (Bernacchioni, Ciarmela, et al. 2021).

In agreement to the pivotal role of S1P signaling in leiomyoma, the overexpression of SK1 in rat leiomyoma cells resulted in increased proliferation and augmented levels of the cell cycle regulator cyclin D1 (Jeng et al. 2007). Moreover, in the same cellular model, Raymond et al. demonstrated that the downregulation or pharmacological inhibition of SK1 significantly reduced the antiapoptotic effect of endothelin (Raymond et al. 2006). The exogenous administration of S1P was also shown to inhibit apoptosis beside increasing the expression of COX2 in rat leiomyoma cells (Raymond et al. 2006).

Our data highlights S1P signaling as a crucial driver of leiomyoma progression, stimulating cell proliferation and fibrosis in leiomyoma but not in myometrial cells. It has been previously shown that the content of S1P and other sphingolipids in human fibroids and pair-matched healthy uterus tissue remains constant (Paweł et al. 2013). However, it should be taken into consideration that not the total variations but rather very localized alterations of S1P levels,

restricted at a specific district of the membrane, are crucial to activating a specific receptor isoform and evoking a biological outcome. Moreover, similarly to our previous study on myoblasts (Cencetti et al. 2010), our recent findings on the increased expression of $S1P_2$, $S1P_3$ and $S1P_5$ in leiomyoma tissue compared to the adjacent myometrium (Bernacchioni, Ciarmela, et al. 2021), support the hypothesis that the remodeling of S1PR expression in leiomyoma might be responsible for the switch of the final biological response evoked by S1P. Unfortunately, our attempt to show any differences in S1PR protein levels by WB analysis in leiomyoma versus myometrial cells was unsuccessful (data not shown). Since the present work has been performed in immortalized cellular models, additional studies employing primary cell cultures will be required to strongly support the involvement of the sphingolipid and dissect the molecular mechanism implicated in the promotion of growth and fibrosis of leiomyoma.

In conclusion, the present data obtained in leiomyoma cells show that S1P increases proliferation through $S1P_2$, acting via ERK1/2 and ERM pathways, and exerts a profibrotic role through S1PR. These results extend the knowledge of the molecular mechanism underlying uterine leiomyoma development and fibrosis, reinforcing the pathogenetic role of S1P and supporting the rationale for a potential treatment targeting the S1P signaling pathway.

5 . Paper 4

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

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5.1 Material and method

5.1.1 Material

Immortalized human endometriotic epithelial 12Z cell line and the Applied cell extracellular matrix were obtained from Applied Biological Materials Inc. (Richmond, BC, Canada). Bradford's reagent (Blue Coomassie G250), Tris/Glycine SDS buffer, PVDF membranes for the Trans-Blot Turbo instrument, EveryBlot Blocking Buffer, and chemiluminescent (ECL) reagents were purchased from Bio-Rad (Hercules, California, 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 (BSA), TRI-Reagent, 30% acrylamide/bis-acrylamide solution, the scramble and specific siRNAs for $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$, the ReBlot Plus Strong Antibody Stripping Solution, the specific ezrin inhibitor NSC668394, the specific 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 as well as the horseradish peroxidase conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). The goat anti-transgelin primary antibody was purchased from Everest (Bicester, UK). Primary rabbit antibodies against the phosphorylated form of ERK 1/2 and ezrin-radixin-moesin (ERM) were purchased from Cell Signalling (Danvers, Massachusetts, USA). The rabbit anti-$S1P_3$ primary antibody was obtained from Abnova Antibodies (Walnut, California, USA). 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 and the "UltraVision LP Detection System" were purchased from Thermo Fisher (Waltham, Massachusetts, USA).

5.1.2 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). Following the described instruction at section 2.1.2.

5.1.3 Immunohistochemistry (IHC)

Tissue samples were fixed with formalin and paraffin embedded. Serial 3 mm sections were obtained from each block, and subsequent slides were stained for IHC analysis of $S1P_3$ (1:75; Abcam [Cambridge, UK]) and Masson Trichrome staining. IHC was performed as described in section 2.1.6.

5.1.4 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 colours 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 had obtained using a Nikon DSF12CCD 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 ×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.

5.1.5 Cell Culture and Treatment

As described in section 2.1.3, when requested, cells were pre-incubated with pharmacological inhibitors 1 h before S1P treatment.

5.1.6 Cell Transfection

Endometriotic epithelial cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX, according to the manufacturer's instructions as described in section 2.1.4.

5.1.7 Quantitative real time PCR (qPCR)

As described in section 2.1.5.

5.1.8 Western Blot Analysis

As described in section 2.1.7.

5.1.9 Statistical Analysis

As described in section 2.1.10

5.2 Results

5.2.1 $S1P_3$ levels as well as fibrosis extent are augmented in endometriotic lesions

The protein expression level of $S1P_3$, previously demonstrated to be increased at mRNA level both in OMA and DIE (Bernacchioni, Capezzuoli, et al. 2021), was quantified by IHC in OMA (n=8) and DIE of different localizations such as urological (n=5), gastrointestinal (n=6), posterior (n=4) as well as in the endometrium of healthy women (CTR, n=10). Representative images, shown in Figure 5-1, (left panel), depict that $S1P_3$ staining was detected, in both endometriotic epithelial and stromal cells, mostly in cytoplasm and membranes. Interestingly, the levels of $S1P_3$ were significantly augmented in all the analyzed glandular sections of endometriotic lesions in respect to control endometrium, in the favour of an increased signalling of the bioactive sphingolipid S1P through $S1P_3$ in endometriosis (Figure 5-1, left panel).

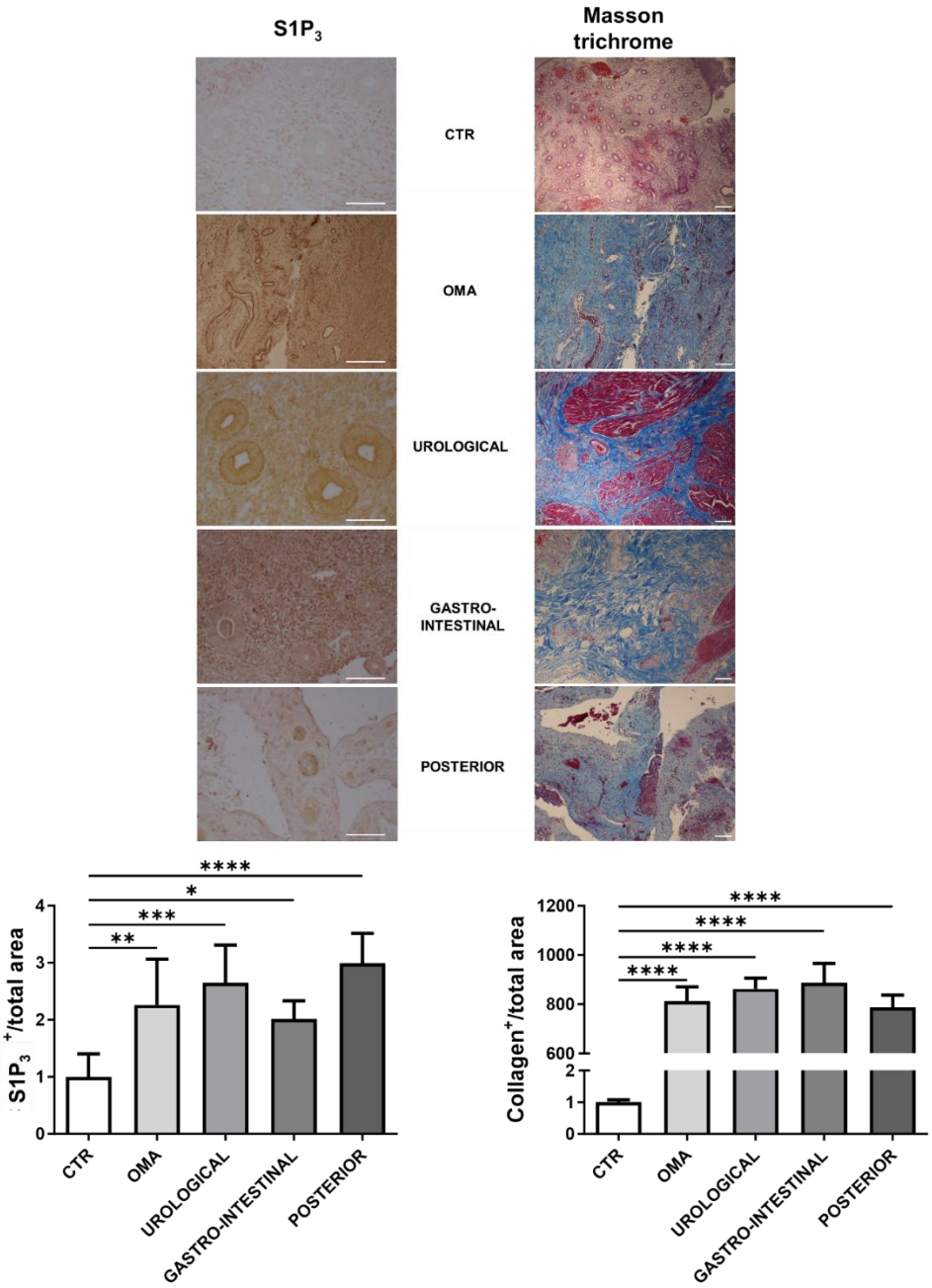

Figure 5-1. **$S1P_3$ levels as well as fibrosis extent are augmented in endometriotic lesions.** Immunohistochemical analysis of $S1P_3$ expression and Masson's trichrome stain in endometriotic lesions (n=8 OMA, n=5 urological, n=6 gastro-intestinal, n=4 posterior) and in the endometrium of healthy women (n=10 CTR). Magnification: 10X for $S1P_3$, scale bar: 200 µm,; 4X for Masson's trichrome stain, scale bar: 100 µm. Results illustrated in the left histogram represent the ratio (mean ± SD) between $S1P_3$ positive area and the total glandular area considered in the analysis and are reported as fold change over the endometrium of healthy women (set as 1). Results illustrated in the right histogram represents 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 by One-way ANOVA (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

In addition, Masson's trichrome staining was performed in the same samples and the representative images reported in highlight that the extent of fibrosis, quantified by blue-stained collagen fibers, is almost absent in the control endometrium whereas is dramatically augmented in all the analyzed endometriotic lesions. Linear regression analyses indicated that the staining levels of $S1P_3$ positively correlated with the extent of fibrosis (Pearson correlation coefficients: r=0.89, *p<0.01 for OMA; r= 0,72 *p<0.01 for DIE), demonstrating the occurrence of a strong correlation between $S1P_3$ and endometriosis-associated fibrosis.

5.2.2 S1P induces EMT/fibrosis in endometriotic epithelial cells

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/EMT was assessed by WB analysis performed in cells treated or not with 1 µM S1P for 48 h. Results shown in Figure 5-2 proved that S1P induced a strong increase in the expression of the EMT markers N-cadherin, vimentin, SNAI-1 and of 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/fibrosis, we studied whether the bioactive sphingolipid was able to activate ERM or ERK1/2, known to be involved in fibrosis and EMT as well as other biological responses (M.-J. Chen et al. 2014; Haynes et al. 2011; X. Chen et al. 2014). As reported in Figure 5-3A, S1P rapidly and transiently activated ERM and ERK1/2 of approximatively 4- and 3-fold, respectively, at the peak of activation (1 min challenge with the sphingolipid).

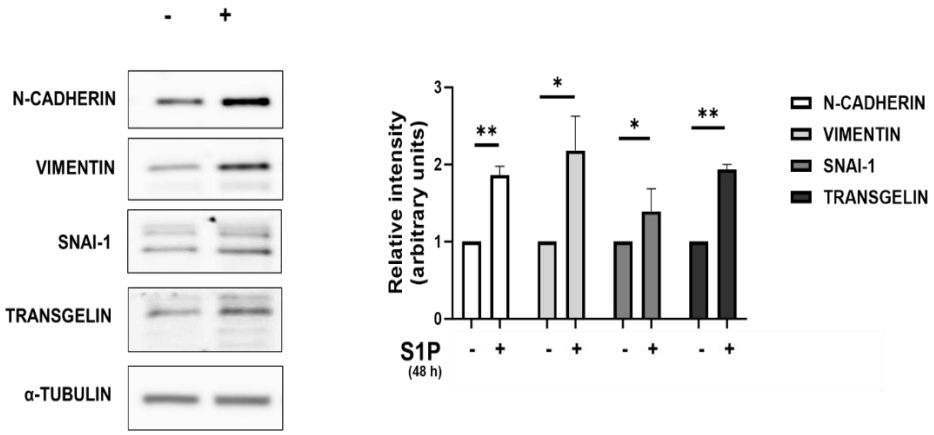

Figure 5-2. **S1P induces EMT/fibrosis in endometriotic epithelial cells.** Endometriotic epithelial cells were treated with 1 µM S1P for 48 h. Protein lysates were analyzed by SDS-PAGE electrophoresis and Western

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<0.05, **p<0.01).

5.2.3 Role of S1PR in S1P-induced activation of ERM and ERK1/2

We next examined whether S1P-induced activation of ERM and ERK1/2 was mediated by S1PR. For this purpose, all the S1PR isoforms were efficiently silenced by RNA interference with specific siRNAs (Figure S 5-1). Of note, only the selective knocking-down of $S1P_3$ significantly reduced the activation of ERM and ERK1/2 elicited by S1P. Conversely, the downregulation of $S1P_1$, $S1P_2$, $S1P_4$ and $S1P_5$ did not affect the S1P-induced action (Figure 5-3B).

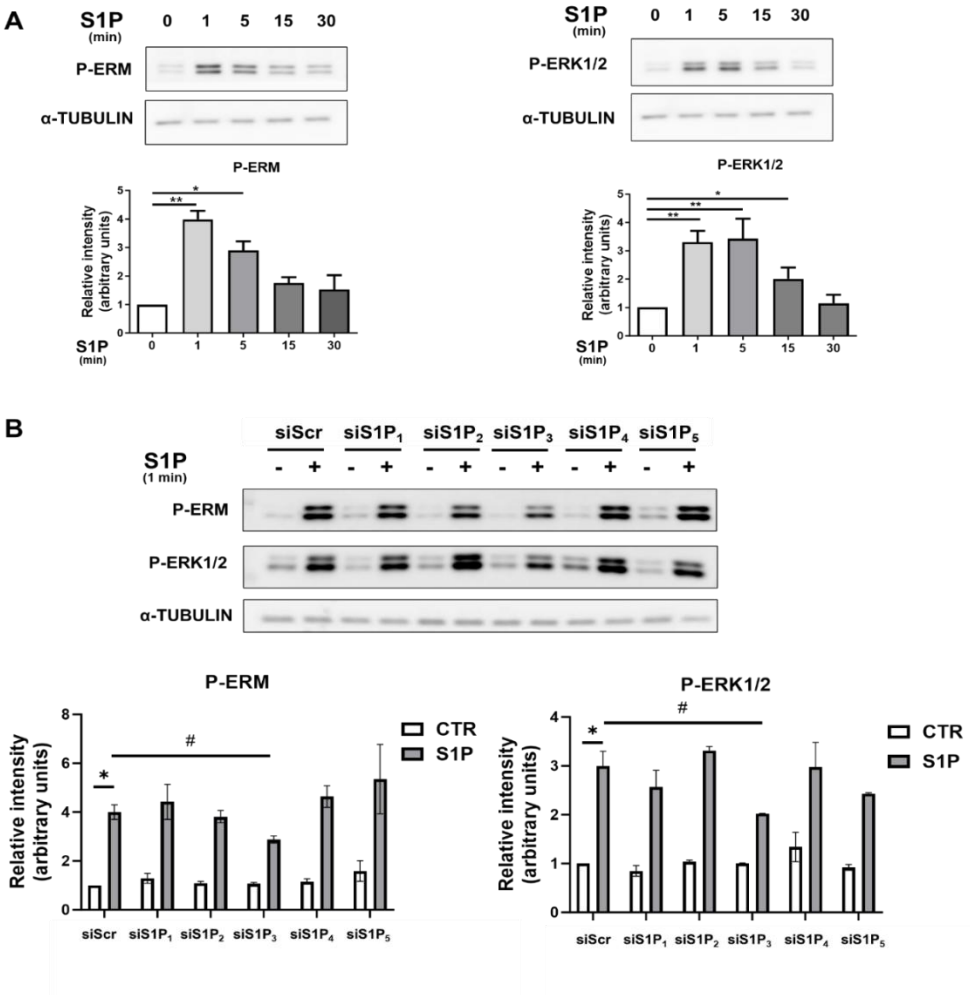

Figure 5-3**. Role of S1PR in S1P-induced activation of ERM and ERK1/2. (A)** Endometriotic cells were overnight serum-starved and then treated with 1 μM S1P for the indicated time intervals (1-30 min). Protein lysates

were analyzed by SDS-PAGE electrophoresis and Western blotting, using specific anti-P-ERM, anti-P-ERK1/2, 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 (*$p<0.05$; **$p<0.01$). **(B)** Endometriotic cells were transfected for 30 h with a non-specific siRNA (scrambled, Scr) or with specific siRNAs for $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ or $S1P_5$ before being treated with 1 μM S1P for 1 min. Protein lysates were analyzed by SDS-PAGE electrophoresis and Western blotting, using specific anti-P-ERM, anti-P-ERK1/2, 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). S1P activated P-ERM and ERK1/2 in a statistically significant manner (*$p<0.05$). The effect of $S1P_3$ down-regulation on S1P induced ERK1/2 and ERM activation is statistically significant by two-way ANOVA, followed by Bonferroni's post hoc test (#$p<0.05$).

5.2.4 $S1P_3$ mediates S1P-induced EMT/fibrosis via ezrin/ERK1/2

Next, to investigate if these signalling pathways activated by S1P through $S1P_3$ are implicated in the profibrotic effect of the bioactive sphingolipid, endometriotic cells were pre-incubated with NSC668394 (2.5 μM) or U0126 (5 μM), which specifically inhibit ezrin and ERK1/2, respectively, before S1P challenge. As shown in Figure 5-4A, the blockade of ERK1/2 or ezrin activation strongly reduced the increase of EMT/fibrosis markers expression brought about by S1P in endometriotic cells. These data suggest a crucial role for the $S1P_3$-dependent activation of ERK1/2 and ezrin in the induction of EMT/fibrosis triggered by S1P. In accordance, WB analysis shown in Figure 5-4B demonstrated that the downregulation of $S1P_3$ 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.

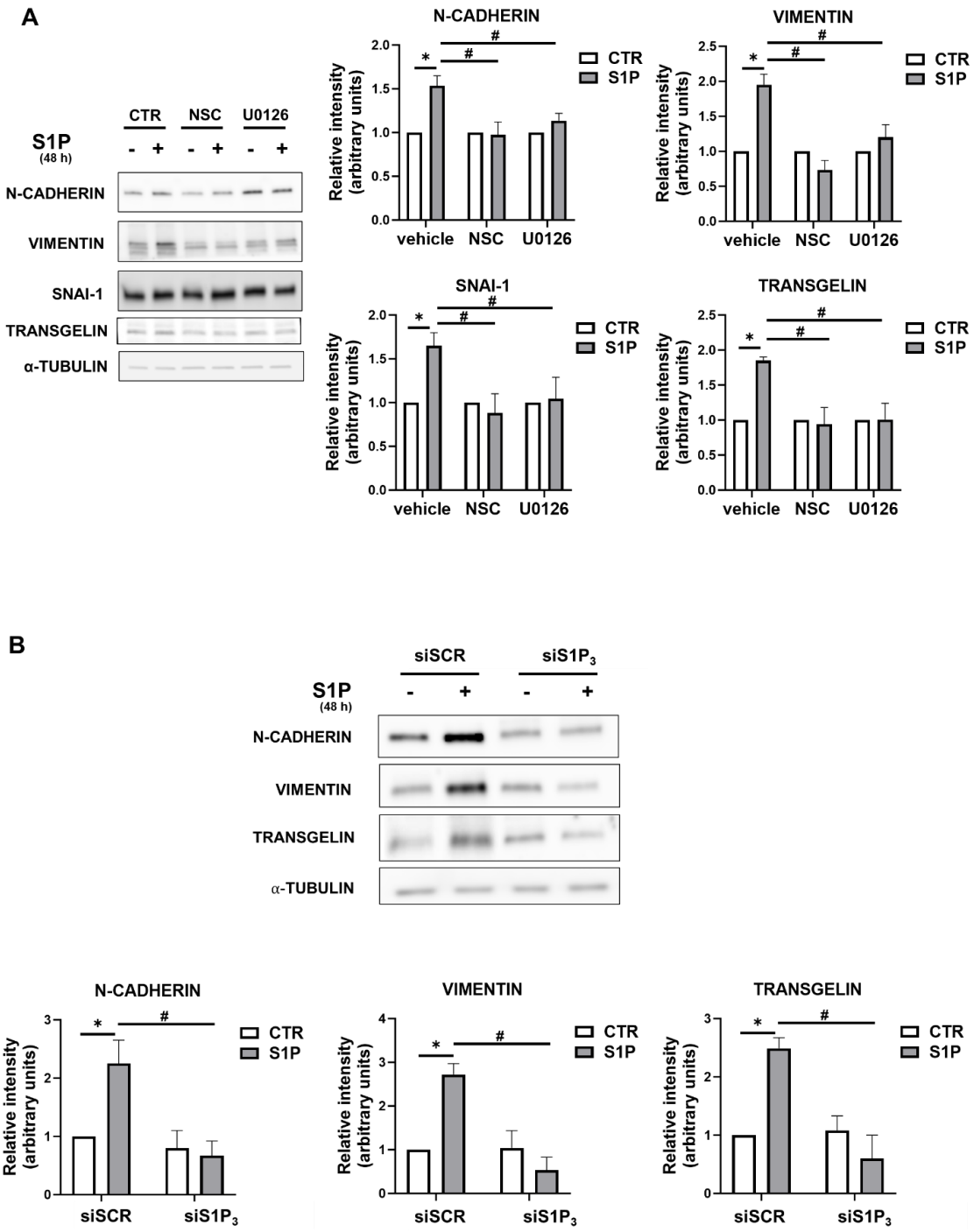

Figure 5-4. **S1P$_3$ mediates S1P-induced EMT/fibrosis via ezrin/ERK1/2**. **(A)** Epithelial endometriotic cells were pre-incubated for 1 h in the presence or absence of NSC668394 (2.5 μM, NSC) and U0126 (5 μM), before being stimulated for 48 h with 1 μM S1P. Protein lysates were analyzed by SDS-PAGE 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). S1P significantly induces the expression of EMT/fibrotic markers (*$p<0.05$). The pharmacological inhibition of phosphorylation of ERK1/2, by U0126, or ezrin, by NSC668396, significantly affected the fibrotic effect of S1P, two-way ANOVA followed by Bonferroni's post hoc test (#$p<0.05$). **(B)** Endometriotic cells were transfected for

30 h with a non-specific siRNA (scrambled, SCR) or with siRNA specific for $S1P_3$ before being treated with 1 μM S1P for 48 h. Protein lysates were analyzed by 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). S1P elicited the expression of vimentin, N-cadherin and transgelin in a statistically significant manner (*$p<0.05$). The effect of $S1P_3$ down-regulation on S1P-induced expression of EMT/fibrotic markers is statistically significant by two-way ANOVA, followed by Bonferroni's post hoc test (#$p<0.05$).

5.2.5 Supplementary Figures

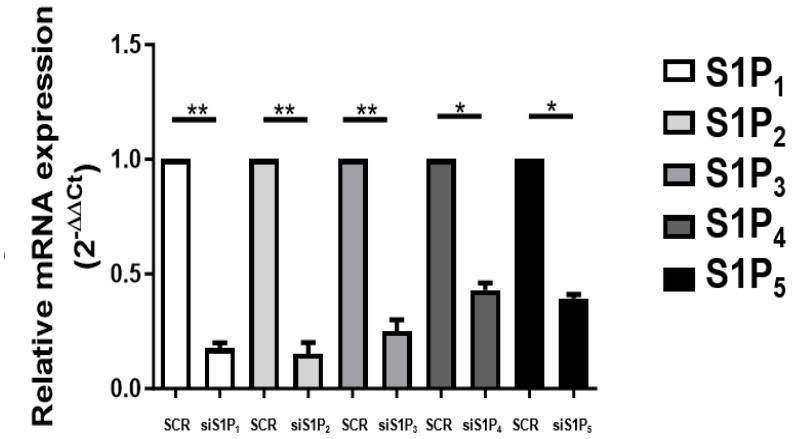

Figure S 5-1. Endometriotic epithelial cells were transfected for 30 h with a non-specific siRNA (scrambled, SCR) or with specific siRNAs for $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ or $S1P_5$ and RT-PCR analysis was performed using TaqMan Gene Expression Assay probes specific for human *S1PR1, S1PR2, S1PR3, S1PR4 or S1PR5*. Results, analyzed with the $2^{-\Delta\Delta Ct}$ method, were normalized to β-actin housekeeping gene. The effect of gene down-regulation was statistically significant by Student's t-test (*$p<0.05$, **$p<0.01$).

5.3 Discussion

Fibrosis and EMT are emerging as key processes in the pathophysiology of endometriosis, being involved in lesion formation, pain and infertility (Zondervan et al. 2020). 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 (Vannuccini et al. 2022).

Findings reported in the present study provide further support to the crucial involvement of S1P signalling 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 $S1P_3$ 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 signalling with the fibrotic trait of the disease. Moreover, the crucial role of $S1P_3$ in fibrogenesis, via the downstream activation of ERK1/2 and ezrin, in human epithelial endometriotic cells has been here demonstrated here. Indeed, WB analysis demonstrated that the downregulation of $S1P_3$ 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 $S1P_3$ 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 previously established the typical occurrence of fibrosis and myofibroblast activation at the level of endometriotic lesions as well as their significance in the pathophysiology of the condition (Zhang et al. 2016; Vigano et al. 2018; Anaf 2000): consequent pelvic adhesions involving different tissue districts (bladder, ureter, colon) can cause dislocation of the implicated organs and pain (Vigano et al. 2018).
Solid literature evidence supports the view that inflammatory mediators, such as TGFβ, are crucially involved in endometriosis-associated fibrogenesis not only stimulating the deposition of ECM but also mediating EMT (Zhao et al. 2022; Vigano et al. 2018). Multiple studies have shown that S1P is involved in EMT via the regulation of TGFβ1 signalling, thus contributing to fibrosis (Milara et al. 2012; King et al. 2011; Donati et al. 2021). S1P has been also

demonstrated to modulate the levels of MMPs such as MMP-2 and MMP-9, regulating ECM remodelling (Kalhori and Törnquist 2015; Kim et al. 2011). The present study focuses on the role of $S1P_3$ and S1P signaling in the modulation of fibrosis, highlighting that the bioactive sphingolipid S1P induces EMT of human epithelial endometriotic cells causing a significant increase of the markers N-cadherin, vimentin, SNAI-1 and transgelin. However, taking into consideration the pivotal role of FMT in fibrogenesis, future studies will be required to deeply investigate the role of $S1P_3$ and its signaling in the stromal endometriotic compartment.

The signalling of the bioactive sphingolipid is involved in the etiopathogenesis of multiple disease states, such as cancer, inflammation and fibrosis (Donati et al. 2021; Maceyka et al. 2012; Cartier and Hla 2019; Yanagida and Hla 2017). Of note, the levels of S1P are augmented in the peritoneal fluid of women with endometriosis (Ono et al. 2021). In addition, the signalling and the metabolism of the bioactive sphingolipid is deeply dysregulated at the level of endometriotic lesions (Bernacchioni, Capezzuoli, et al. 2021; Santulli et al. 2012). Moreover, we recently showed that S1P induces a ROS-mediated proinflammatory response in human endometrial stromal cells (Seidita et al. 2023), further suggesting a crucial role of S1P in the endometriosis-associated inflammatory scenario.

Notably, the $S1P_3$ isoform has been crucially linked to the development of fibrosis in different tissues such as skeletal muscle, lung, kidney and heart (Zhang et al. 2018; Bruno et al. 2015; Xin et al. 2004; Takuwa et al. 2010; Cencetti et al. 2010). The trans-differentiation of myoblasts into myofibroblasts triggered by TGFβ1 is mediated by SK1 and $S1P_3$ signalling in skeletal muscle myoblasts: increased levels of the cytokine are responsible for the upregulation of SK1 and $S1P_3$, which becomes the most expressed receptor, causing a shift of the signalling from myogenic to fibrotic phenotype (Cencetti et al. 2010). $S1P_3$ was shown to mediate the ability of TGFβ1 to induce fibrosis and EMT markers in a model of uterine adenocarcinoma cells (Bernacchioni, Capezzuoli, et al. 2021). In a SK1 transgenic mouse model, $S1P_3$ deletion was shown to inhibit Smad-associated cardiac fibrosis (Takuwa et al. 2010) and in normal lung fibroblasts, the activation of $S1P_2$ and $S1P_3$ was demonstrated to induce the synthesis of ECM (Sobel et al. 2013).

ERK1/2 crucially operates in intracellular signalling pathways by phosphorylating specific serine/threonine residues of target molecules in response to a broad array of stimuli. Although the effects of ERK1/2 activation are strictly dependent on cell type and the specific stimulus, typically entail the regulation of cellular proliferation, survival, and differentiation but also carcinogenesis and fibrosis (X. Chen et al. 2014; Mebratu and Tesfaigzi 2009).

Activation of ERK1/2 was reported to mediate TGFβ2-and HGF-induced EMT in human epithelial lens cells and in a mouse model of liver carcinogenesis (Tanahashi et al. 2013). Notably, ERK1/2 activity has been found to be abnormally regulated in the endometrial tissue of women with endometriosis (Klemmt et al. 2006; Arosh and Banu 2019). Ezrin belongs to the ERM family of proteins that, localized beneath the plasma membrane of cellular protrusions (Bretscher et al. 1997), act as linkers between the plasma membrane and the actin cytoskeleton and are involved in cell adhesion and membrane ruffling (Tsukita and Yonemura 1997). Eutopic and ectopic endometrial tissues from women affected by endometriosis have been demonstrated to be characterized by higher levels of ezrin and phospho-ezrin by IHC and WB analysis (Ornek et al. 2008). Recently, ezrin has been shown to regulate cell migration and invasion in TGFβ1-induced EMT in human alveolar epithelial cells (M.-J. Chen et al. 2014). In the present study, we showed that S1P, via $S1P_3$, was able to rapidly and transiently increase the activation of ERK1/2 and ezrin and when their activation was pharmacologically blocked, the sphingolipid was unable to augment the expression levels of the fibrotic/EMT markers.

Taken together, these data expand our knowledge on the molecular mechanisms responsible for endometriosis pathogenesis and reinforce the rationale for the exploitation of S1P signalling as innovative therapeutic target for its treatment. Indeed, the signalling axis of the bioactive sphingolipid has been already employed in approaches based on S1PRs modulators, such as fingolimod and ozanimod, for the treatment of multiple sclerosis and other immune syndrome (Park and Im 2017; Brinkmann et al. 2010).

The present data showed for the first time a positive correlation between the extent of fibrosis and $S1P_3$ immunostaining levels in different types of endometriotic lesions (OMA and DIE). The crucial role of $S1P_3$ 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, $S1P_3$ may be proposed as a possible target to counteract the inflammation-associated fibrotic trait of endometriosis.

6 . General Conclusion

Endometriosis is a complex, chronic inflammatory disorder that still lacks definitive therapeutic options (Zondervan et al. 2020). In recent years, the S1P signaling axis has been increasingly associated with the pathogenesis of endometriosis (Ono et al. 2021; Yoshino et al. 2019; Santulli et al. 2012; Bernacchioni, Capezzuoli, et al. 2021). ECS alternations have been observed in endometriosis, but its precise contribution to disease onset and progression remains unclear.

In the first paper we have shown that the ECS receptors and metabolic enzymes are present in the endometriotic tissue. In the next step after confirming the expression of the ECS receptors and enzymes in the endometriotic epithelial cells 12Z we have revealed that there is a crosstalk between S1P signaling axis and ECS in which the pro-inflammatory effect of 2-AG in 12Z cells is dependent on $S1P_3$. However, the exact mechanism is yet to be discovered and there is need for more investigations.

Adenomyosis is a benign uterine disorder characterized by the presence of endometrial tissue within the myometrium. Inflammation is an important factor in the pathogenesis of adenomyosis (Zhai et al. 2020; Vannuccini et al. 2017).
In the second paper we provide evidence that the mRNA levels of CB1, CB2 and GPR18 are augmented in adenomyotic tissue in comparison to healthy endometrium. Noteworthy, for the first time we showed the experimental evidence regarding the expression of the receptor GPR18 in adenomyotic lesions. Considering that GPR18 was previously shown to mediate the pro-migratory action of different endocannabinoid agonists in endometrial cells, it could light up a new molecular point to be considered in adenomyosis studies.

Leiomyomas are the most common benign pelvic tumors in reproductive-age women. Their development involves cytokines that regulate inflammation, proliferation, and angiogenesis. Alongside cell proliferation, fibrosis is a key characteristic of these tumors (Islam et al. 2018; Yang et al. 2022).

In the third paper the findings in leiomyoma cells demonstrate that S1P promotes cell proliferation through $S1P_2$ by activating the ERK1/2 and ERM pathways, while it induces fibrosis via S1PR. These results deepen the understanding of the molecular mechanisms driving uterine leiomyoma growth and fibrotic progression, emphasizing the pathogenic role of S1P.

The endometriosis associated fibrosis includes varying degrees depending on the localization of the lesion. The presence of fibrotic tissue inside and surrounding the endometriotic ovarian and peritoneal lesions is the main reason for pelvic pain in endometriosis caused by tissue adhesions, scarring, and anatomic distortions (Vigano et al. 2018; Garcia Garcia et al. 2023). However, the molecular mechanisms underlying the development of the fibrotic phenotype in endometriotic lesions is yet to be fully clear.

In the last paper our data showed for the first time a positive correlation between the extent of fibrosis and $S1P_3$ immunostaining levels in different types of endometriotic lesions (OMA and DIE). The crucial role of $S1P_3$ 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, $S1P_3$ may be proposed as a possible target to counteract the inflammation-associated fibrotic trait of endometriosis.

Collectively, these findings enhance our understanding of the molecular mechanisms involved in the development of endometriosis pathogenesis, highlighting the critical role of $S1P_3$ in mediating the pro-inflammatory action of 2-AG in endometriotic epithelial cells as well as in the process of fibrosis. Considering that ECS receptors were shown to be more expressed in the adenomyosis tissue compared to control, maybe the crosstalk with S1P signaling can be valid also for the pathogenesis of this disease. In the past, S1PR modulators have emerged as promising therapeutics for various immune-mediated diseases, including multiple sclerosis, inflammatory bowel disease and psoriasis, by modulating lymphocyte trafficking and reducing tissue inflammation (McGinley and Cohen 2021). Since we recently demonstrated that $S1P_3$ is involved in the invasive trait of endometriotic cells (Prisinzano et al. 2024; Bernacchioni et al. 2024), the here presented data further support the potential of targeting $S1P_3$ as a novel non-hormonal therapeutic strategy for endometriosis treatment. The data in leiomyoma cells demonstrate that S1P promotes cell proliferation through $S1P_2$ also highlights the importance of the S1PR role thus establishing the rationale for targeting the S1P pathway as a potential therapeutic or diagnostic strategy for uterus pathogenesis including endometriosis, adenomyosis and uterine fibroids.

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