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Sphingosine 1-phosphate: a new player in uterine disorders

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1. INTRODUCTION

1.1 UTERUS

The uterus or womb is the main hormone-responsive secondary sex organ of the female reproductive system in humans (Fig. 1). It is situated within the pelvis, immediately behind, almost overlying the bladder and in front of the sigmoid colon. The human uterus is a pear-shaped, fibromuscular organ and measures approximately 8 cm in length, 4 cm in width and 5 cm in depth in the normal non-pregnant state. It is held in position inside the pelvis by several ligaments, including the utero-ovarian ligament, round ligament, broad ligament, cardinal ligaments, and uterosacral ligaments. The uterus can be divided anatomically into four principal regions: the fundus, the wide curved upper surface in which the fallopian tubes connect to the uterus; the corpus (body), the main part of the uterus; the isthmus, the lower, narrow neck area; and the cervix, extends downward from the isthmus until it opens into the vagina. The uterus is composed of three layers, which together form the uterine wall (Guyer, Rajesh, and E. Connor 2020). From innermost to outermost, these layers are the endometrium, myometrium and perimetrium (Fig. 1).

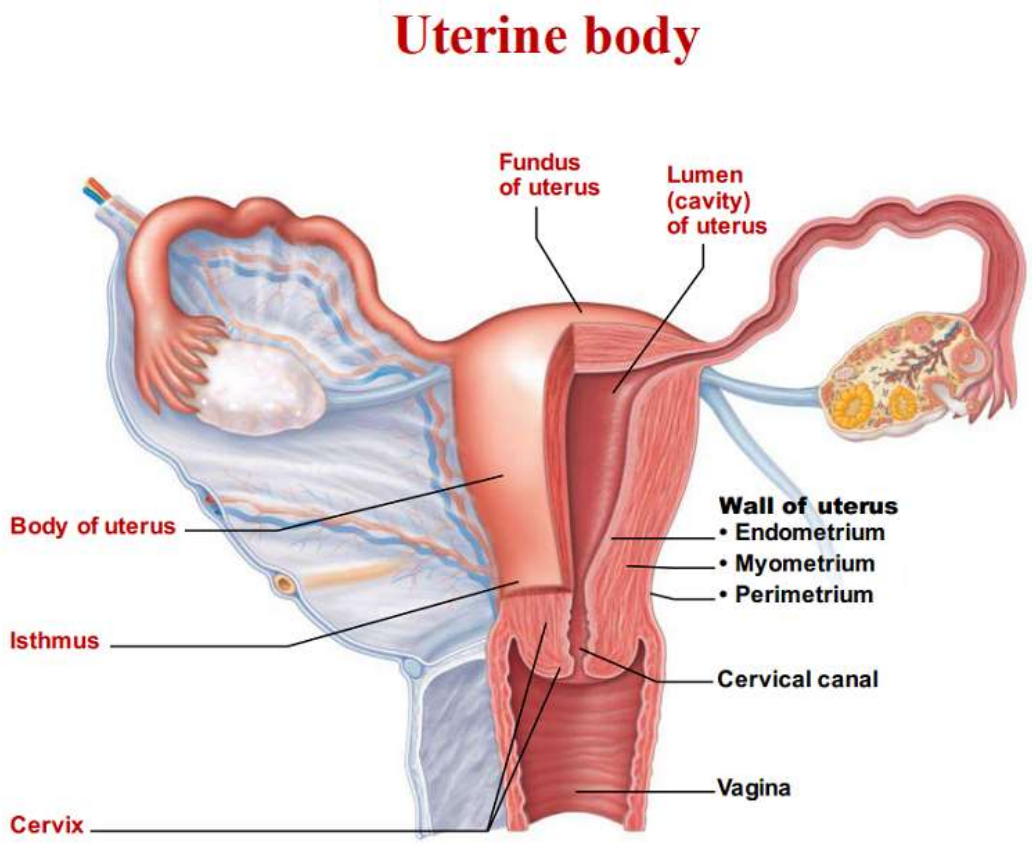

Figure 1 Female reproductive system

The endometrium is a glandular mucous membrane and constitutes the inner epithelial layer of the uterine cavity. It is characterized by a single superficial layer of columnar epithelium containing ciliated and secretory cells, which overlies a stromal layer of variable thickness during the menstrual cycle, containing many tubular endometrial glands that extend deep into the stroma. The main function of the endometrium is to receive the conceptus after fertilisation and to allow the growth of the embryo and fetus (Ferenczy and Bergeron 1991). The myometrium of the uterus is primarily composed of smooth muscle and an innermost layer known as the "junctional zone", that is structurally and functionally different from the outer myometrial area (Brosens, Barker, and de Souza 1998). Finally, the perimetrium is a serous layer of the visceral peritoneum which covers the outer surface of the uterus (Ross and Pawlina 2016).

Uterine disorders are multifactorial alterations of complex and polygenic nature, which often compromise women's fertility. The most common uterine disorders include endometriosis, adenomyosis and uterine myomas.

1.2 ENDOMETRIOSIS

1.2.1 Introduction

Endometriosis is a common, oestrogen-dependent, gynaecological disorder associated with pelvic pain and infertility. Histologically it is defined as the presence of endometrial glands and stroma outside the uterine cavity (Giudice 2010). Endometriosis is a heterogeneous disease with three well-recognized phenotypes: ovarian endometriotic cysts, called endometriomas (OMA), superficial peritoneal lesions (SUP), and deep-infiltrating endometriosis (DIE) (Fig. 2). The latter are defined as lesions with more than 5 mm depth of invasion beneath the peritoneum or infiltration into the muscularis propria of the organs that surround the uterus (Foti et al. 2018). In addition, endometriosis can occur in extragenital locations, like, pleural, diaphragmatic or umbilical (Menni, Facchetti, and Cabassa 2016).

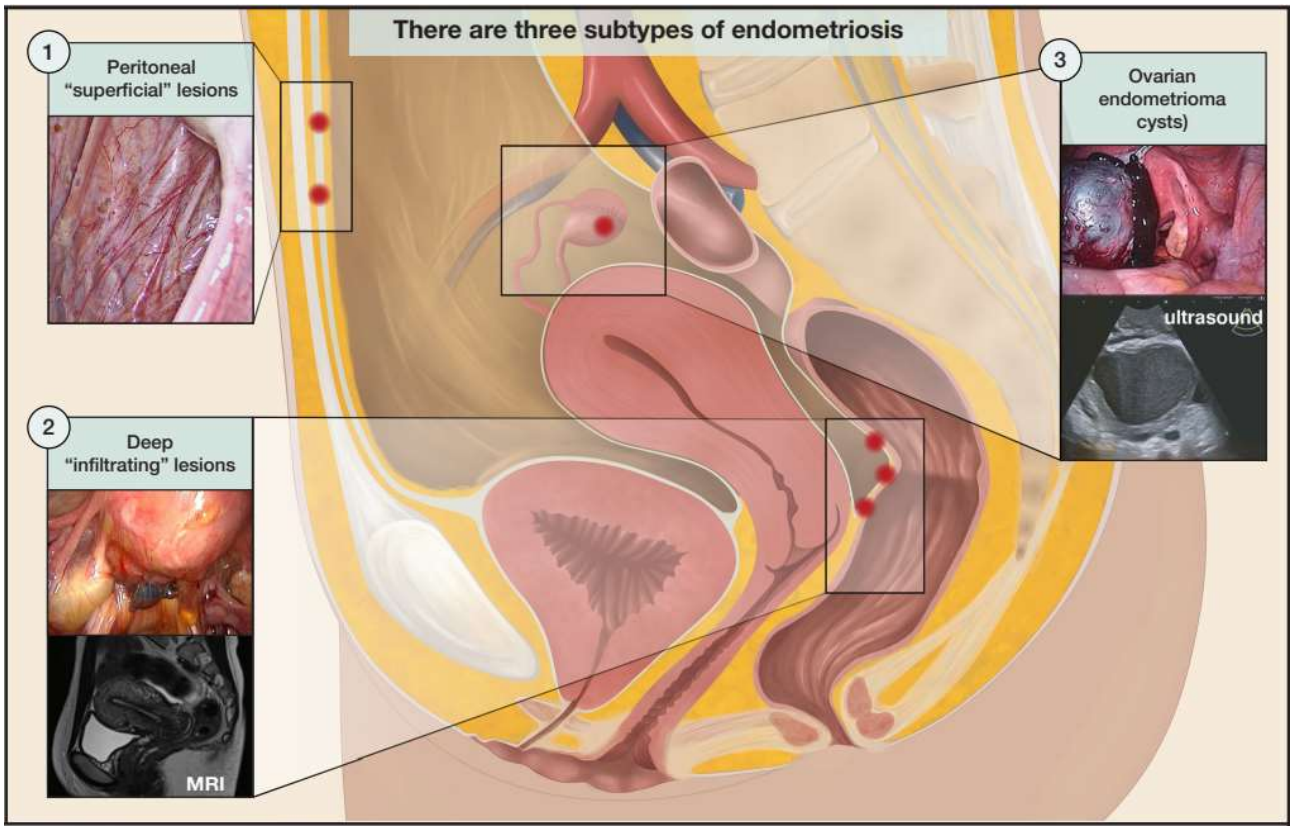

Figure 2 Endometriosis phenotypes (Horne and Saunders 2019)

Endometriosis is a debilitating condition which adversely affects the patient's quality of life (Berkley, Rapkin, and Papka 2005). Clinical symptoms include pelvic pain, dysmenorrhoea, dyspareunia, and infertility (Vercellini et al. 2007; Schliep et al. 2015; Apostolopoulos et al. 2016). In addition, the

disease is associated with depression (L.-C. Chen et al. 2016) and fatigue (Ramin-Wright et al. 2018), thereby leading to a loss of work productivity and causing a significant economic burden (Soliman et al. 2016) (Fig. 3).

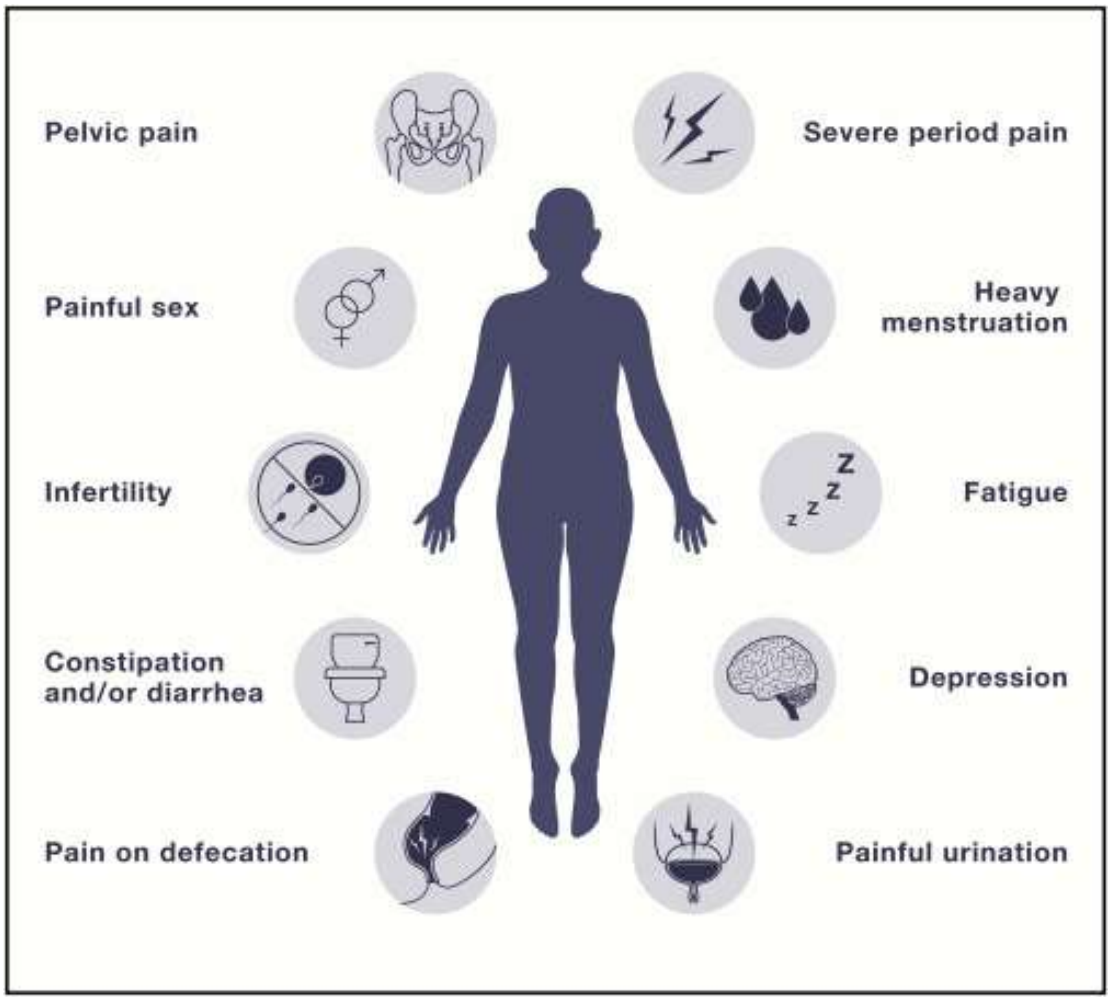

Figure 3 Symptoms of endometriosis (Saunders and Horne 2021)

Given these effects, endometriosis should be considered a public health issue rather than a disease of individuals. The prevalence of endometriosis approaches 6–10% in the reproductive female population. In women with pelvic pain, the frequency reaches 50-80% and up to 50% of women with infertility. However, despite this high prevalence, disease recognition is inadequate, and diagnosis time ranges from 4 to 11 years, with 65% of women being initially misdiagnosed (Greene et al. 2009). The reasons for this delay are various. Endometriosis does not present specific pathognomonic signs and symptoms for an isolated pelvic disorder. Indeed, the disease is characterized by symptoms common to other gynaecological and non-gynaecological conditions. For example, pelvic pain, manifested by a woman suffering from adenomyosis or uterine fibroids, should raise the suspicion of

coexisting endometriosis, as these diseases are commonly associated (Nezhat et al. 2016; Chapron et al. 2017). Functional bowel and bladder disorders and fibromyalgia can also be symptoms that overlap with endometriosis. Furthermore, the current requirement for surgical diagnosis, usually by diagnostic laparoscopy, represents a barrier to early recognition and treatment. The absence of visible lesions or negative histology does not rule out the diagnosis of endometriosis, in fact occult endometriosis has been documented in random peritoneal biopsy samples (Albee, Sinervo, and Fisher 2008; Stegmann et al. 2008). The need for early diagnosis and treatment of the disease cannot be overstated. Early identification and treatment of endometriosis is crucial and facilitated by a shift towards clinical diagnosis instead of relying solely on surgical diagnosis.

Currently, the American Society for Reproductive Medicine (ASRM) system, originally developed in 1985 and revised in 1997, is the global standard used for endometriosis staging ('Revised American Society for Reproductive Medicine Classification of Endometriosis: 1996' 1997, 1996). It is classified into four stages (I, II, III and IV) according to the surgical evaluation of the size, location, and severity of endometriotic lesions and the occurrence of extensions of adhesions (Fig. 4). However, despite widespread implementation, the ASRM system is insufficient because it poorly correlates with pain symptoms, infertility and excludes extra-pelvic lesions.

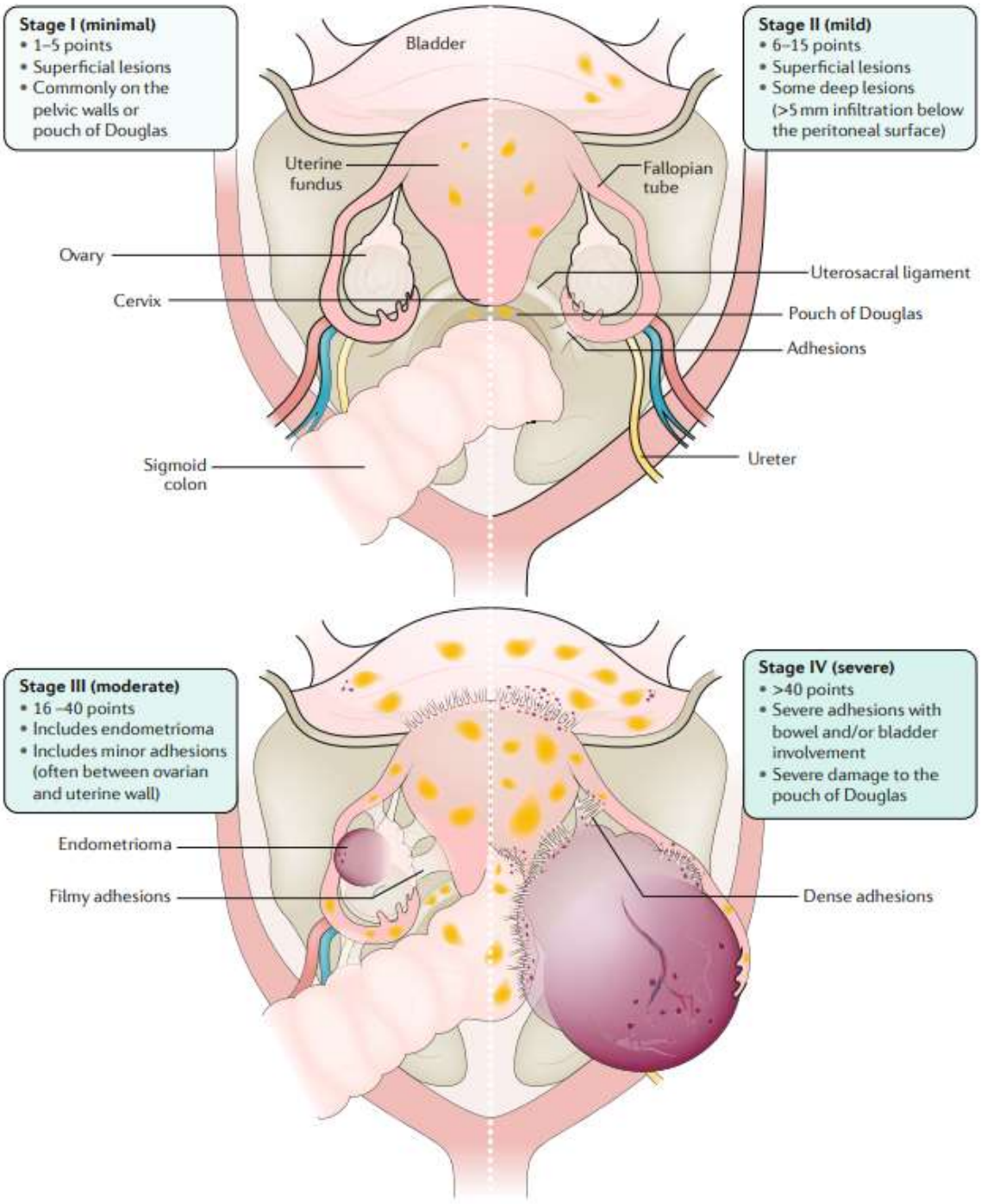

Figure 4 Staging of endometriosis (Zondervan et al. 2018)

1.2.2 Pathogenesis and progression of endometriosis

The most well-accepted pathophysiological hypothesis for endometriosis, is based on Sampson's retrograde menstruation theory, which is observed in the majority of patients (Dastur and Tank 2010) (Fig. 5). In this process, menses transport viable endometrial fragments through the fallopian tubes to the peritoneal cavity, possibly by a pressure gradient originating from dys-synergic uterine contractions, where they can implant, develop, and invade other tissues of the pelvis, generating a suboptimum immune response that does not adequately clear the implants, and resulting in continued survival and growth. This hypothesis is supported by epidemiological evidence, that shows an enhanced risk of endometriosis with increased exposure to menstruation, and asymmetry in the anatomical location of the endometriotic lesions (Missmer et al. 2004). Indeed, the latter tend to have an asymmetrical distribution, which could be explained by the effect of gravity on menstrual flow, the abdominopelvic anatomy and the peritoneal clockwise flow of menses (Bricou, Batt, and Chapron 2008). In addition, in the pelvis, endometriosis is most commonly seen in the posterior compartment and on the left side, while lesions in the abdomen and the thorax are mostly located on the right side. However, whereas the majority of women have retrograde reflux, only 10–15% of women develop endometriosis. Another theory, has been proposed to explain the origin of endometriosis, named "Coelomic metaplasia" (Fig. 5). The latter, first suggested by Mayer and subsequently perfected by Ferguson and colleagues (Ferguson, Bennington, and Haber 1969), provides the transformation of peritoneal mesothelium into glandular endometrium. Recent studies suggest that this process involves the reprogramming of multipotent mesenchymal stem cells, (Figueira et al. 2011) derived from the bone marrow (Du and Taylor 2007) or from an internal niche of the endometrium itself (Gargett and Masuda 2010), which can differentiate into endometrial epithelial and stromal cells in ectopic sites. It's hypothesized that these metaplastic changes occur as a result of hormonal influences, inflammatory processes, or the action of one or more endogenous biochemical or immunological factors, derived from the eutopic endometrium (Laganà et al. 2019). The hypothesis of Coelomic metaplasia may explain endometriosis in the absence of retrograde menstruation; furthermore, it has also been suggested as the origin of the rare cases in which endometriotic lesions occur in sites outside the pelvis, such as, for example, in the chest, pleura, lungs, brain, and in the nasal cavity (Andres et al. 2020). This hypothesis is also supported by sporadic cases, in which male endometriosis has been observed (Taguchi, Enomoto, and Homma 2012); or in the cases of Mullerian agenesis (the congenital malformation in which the Müllerian duct fails to develop) (Troncon et al. 2014). Finally, another important theory proposed to explain the origin of endometriosis, is represented by the hypothesis of metastasis (Fig. 5). According to the latter, endometrial cells and tissue fragments can travel from the

uterine cavity, through the lymphatic system canals and veins, to colonize distant ectopic sites (Halban 1925). Microvascular studies have demonstrated the flow of lymph from the uterine body into the ovary, making possible a role for the lymphatic system in the aetiology of OMA. In addition, the presence of endometriotic lesions within the lymph nodes has been documented in a baboon model of induced endometriosis (Hey-Cunningham et al. 2011), and in 6-7% of women at lymphadenectomy (Javert 1952). This hypothesis therefore best describes the rare occurrence of extrapelvic endometriosis in women.

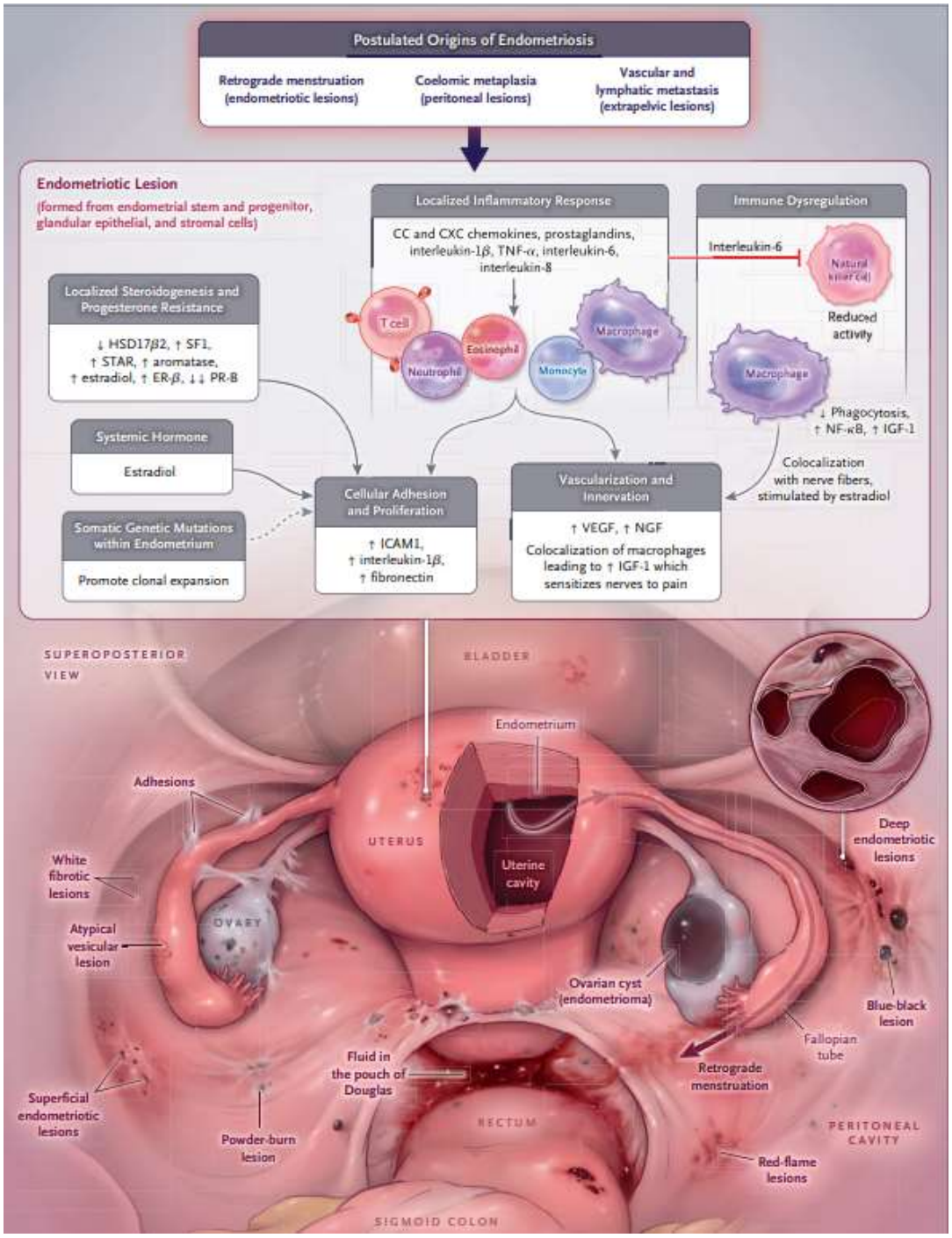

Figure 5 Origin of Endometriosis (Zondervan, Becker, and Missmer 2020)

The development of endometriosis involves interacting endocrine, proinflammatory, immunological and fibrotic processes. The tissue microenvironment controls these phenomena, and its regulation is influenced by various hormonal and cellular factors. Among these, oestrogens are the key mediators of endometrial cell growth. Indeed, an enhanced expression of steroidogenic factor 1 (SF1), a transcription factor that induces the gene expression of aromatase, the enzyme responsible for the conversion of androstenedione to estrone and of testosterone to estradiol, has been shown in endometriotic stromal cells. In addition, a lack of expression of hydroxysteroid 17β dehydrogenase 2, which normally oxidizes estradiol to its less potent metabolite, estrone, was observed in ectopic endometrial implants and ectopic epithelia. High local concentrations of estradiol and the upregulation of ERα and ERβ receptors activate a network of genes such as *GREB1*, *MYC* and *CCND1*, that regulate cellular mitogenesis (Pellegrini et al. 2012). In a mouse model it was demonstrated that increased ERβ activity in endometriotic lesions was able to promote endometriotic tissue growth in three different ways: by reducing tumour necrosis factor (TNF)-induced apoptosis, increasing cell adhesion and proliferation mediated by IL-1β, and increasing the epithelial-mesenchymal transition (EMT) (Han et al. 2015). Furthermore, the dysregulation of the progesterone receptors (PRs) and the alteration of their signalling pathways in eutopic and ectopic endometrium, results in progesterone resistance in up to 30% of women with endometriosis (Al-Sabbagh, Lam, and Brosens 2012). The progesterone resistance, in turn, determinates the inhibition of oestrogen-dependent epithelial-cell growth and dysregulation of endometrial decidualization (Zondervan, Becker, and Missmer 2020). Endometriosis is characterized by an intense localized immune and inflammatory response, with the production of cytokines, chemokines and prostaglandins. The inflammatory response involves monocytes, T cells, and eosinophils attracted by CC chemokines; and macrophages and neutrophils, attracted instead by CXC chemokines (Reis, Petraglia, and Taylor 2013). Despite the high production of chemokines, and consequently, an increase in the recruitment of local macrophages, the power of their scavenger function and phagocytotic potential appears to be inhibited (Lessey, Lebovic, and Taylor 2013). The activity of natural killer cells is impaired in women with endometriosis, which can contribute to immune evasion of endometrial cells (Kang et al. 2014). Moreover, an exacerbated expression of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), a key regulator of the chemokine gene and protein expression, has been demonstrated in cultured endometriotic stromal cells and peritoneal macrophages isolated from women with endometrioma (X.-Q. Wang et al. 2010). Cytokine production is further enhanced by reactive oxygen species and by the activation of other inflammatory pathways, mediated by an extracellular signal-regulated kinase (ERK1/2), mitogen-associated kinase (MAPK) and Jun N-terminal kinase (JNK) (Beste et al. 2014; McKinnon et al. 2016). Moreover, an increase in the concentration of type 17

helper T cells was observed in the peritoneal fluid of women with endometriosis, resulting in increased expression of interleukin-17 and induction of chronic inflammation (Symons et al. 2018). Finally, a key feature of endometriosis is the presence of fibrotic tissue in and around the lesions, that contributes to the classic symptoms related to endometriosis like pain and infertility. Despite this, the molecular mechanisms responsible for the development of fibrosis in endometriosis are not yet fully clarified. Activated platelets, macrophages, ectopic endometrial cells and sensory nerve fibres have been shown to promote fibrogenesis in endometriotic lesions by inducing the release of factors that favour EMT, fibroblast-to-myofibroblast transdifferentiation (FMT), collagen deposition, and fibrosis (Viganò et al. 2020). Zhang et al (Q. Zhang, Duan, Olson, et al. 2016; Q. Zhang, Liu, and Guo 2017) demonstrating that endometriotic lesions are wounds that undergo repeated tissue injury and repair (TIAR) mechanisms, leading to fibrosis. This process involves the development of "leaky" blood vessels, and the extravasation and aggregation of platelets, which then release transforming growth factor (TGF) -β1 and activate the TGF-β1 signalling pathway / Smad3 (S.-W. Guo, Ding, and Liu 2016), leading to fibrosis in endometriotic lesions. Macrophages play a key role in the development of tissue fibrosis (Capobianco and Rovere Querini 2013; Johan et al. 2019). In humans, they have been shown to produce critical mediators for profibrotic phenomena, such as TGF-β1, vascular endothelial growth factor (VEGF), TNF-α, IL-1 and IL-6 (Ruiz et al. 2015), which in turn can induce EMT, FMT, and smooth muscle metaplasia (SMM) in endometriotic epithelial and stromal cells, resulting in enhanced contractility, collagen deposition and fibrosis. Finally, recent studies have shown the importance of sensory nerve fibres in the endometriosis-related fibrosis process (Xishi Liu, Yan, and Guo 2019), by promoting EMT and FMT with consequent production of collagen and adhesions in endometriotic lesions. A critical role of TGF-β has been demonstrated in the initiation and progression of fibrosis in OMA (Young et al. 2017; Shi et al. 2017). Endometriotic cells can synthesize TGF-β1, which accumulates in the surrounding ovarian tissue, disorganizing the extracellular matrix (ECM) and promoting fibrosis around OMA. Furthermore, an important correlation has been found between lactate and TGF-β1 concentrations in ectopic lesions, suggesting that TGF- β1 can regulate changes in cellular metabolism that are capable of promoting ectopic cell survival (Young et al. 2014). Another study has shown that Activin A, a member of the TGF-β family, facilitates the invasion of endometrial stromal and epithelial cells in an *in vitro* model of the peritoneum (Ferreira et al. 2008), suggesting its possible involvement in the pathogenesis of endometriosis. Activin A also plays a critical role in the differentiation of endometrial stem cells toward the myofibroblast phenotype (Z. Zhang et al. 2019). Finally, in a mouse model of endometriosis, an antibody against Activin A significantly inhibited the excessive deposition of collagen and the expression levels of collagen I (Col-I), αSMA and connective tissue growth factor

(CTGF) in ectopic lesions, providing the experimental basis for the treatment of fibrosis-related endometriosis through manipulation of Activin A signalling.

1.2.3 Treatments

The goal of endometriosis treatment is to suppress the growth of the lesions, treat pain, and ideally treat the systemic effects of the disease (Reis, Petraglia, and Taylor 2013; Brichant et al. 2021). According to the National Institute for Health and Care Excellence guideline, the first-line treatment consists of non-steroidal anti-inflammatory drugs (NSAIDs), typically in combination with progestin-based therapy (National Guideline Alliance (UK) 2017). Second-line treatments include GnRH agonists that reduce oestrogen levels to post-menopausal concentrations. NSAIDs suppress the function of cyclooxygenase enzymes, thus decreasing prostaglandin concentrations and inflammation. Progestins include: medroxyprogesterone, norethisterone, and dienogest (Buggio et al. 2017), and they negatively influence cell proliferation, inflammation, neovascularization, and neurogenesis in endometriosis. However, up to a third of patients do not respond to first-line therapies because of progesterone resistance or side effects. GnRH agonists, by lowering GnRH pulsatility, inhibit the gonadotrope axis, preventing oestrogen stimulation on ectopic glands. This leads to post-menopausal levels of oestrogen, leading to gradual bone loss and/or severe vasomotor symptoms which restrict their use to 6 months without additional therapy (Olive 2008). For patients who do not respond to hormonal therapy, novel drugs (such as GnRH antagonists (Taylor et al. 2017), selective oestrogen or progesterone receptor modulators, anti-angiogenic drugs, and antioxidants,) are promising new treatments, even if they require further evaluation (Ferrero, Evangelisti, and Barra 2018). Surgery is a therapeutic option for endometriosis to efficiently treat pelvic pain and infertility. Two surgical modalities should be considered. The first is conservative surgery, defined as the exeresis of endometriotic lesions without removing the uterus and/or the ovaries. The second modality is the definitive surgery, which includes the excision of all the endometriotic lesions associated with concurrent hysterectomy with or without oophorectomy. Although surgery remains an important strategy for managing endometriosis, many limitations should be considered by health-care professionals. Laparoscopic surgery for treatment of endometriosis is not curative, since 40–45% of women suffer from recurrence of pain. The probability of needing repeated surgery in 2 years is 15–20%, reaching 50% within 5–7 years (Hamdan et al. 2015). Of note, surgery can have major complications, especially in cases of DIE surgery (for example, postoperative infection, neurogenic bladder and bowel dysfunction), which could affect the quality of life of patients (Rizk et al. 2015).

Also of importance is the potential negative effect on the ovarian reserve after OMA laparoscopic cystectomy (Raffi, Metwally, and Amer 2012).

1.3 ADENOMYOSIS

1.3.1 Introduction

Adenomyosis is a compound word, etymologically deriving from the Greek terms αδénas (αδένας), meaning gland, and mís (μυς), meaning muscle, and regarding to a pathological state of the muscle involving glands. Rokitansky, in 1860, was the first to identify the presence of endometrial glands and stromal cells inside the myometrium (adenomyosis) and outside the uterine cavity (endometriosis), calling the two conditions respectively "internal endometriosis" and "external endometriosis " (Rokitansky 1860). Although the term “adenomyosis uteri” was coined in 1925 (Frankl 1925), the present definition is based on a 1972 publication, when Bird et al. defined adenomyosis as “benign invasion of endometrium into the myometrium, producing a diffusely enlarged uterus which microscopically exhibits ectopic, non-neoplastic, endometrial glands and stroma surrounded by hypertrophic-hyperplastic musculature” (Bird, McElin, and Manalo-Estrella 1972). Today, adenomyosis is commonly described as an estrogen-dependent uterine disorder, in which endometrial tissue invading the myometrium to a depth of at least 2.5 mm at the time of histological diagnosis, and frequently surrounded by hyperplastic and hypertrophic smooth muscle (Stratopoulou, Donnez, and Dolmans 2020). Adenomyosis may present as focal lesion when a nodular collection is identified, or as diffuse when glands and stroma are dispersed within the myometrium (Exacoustos et al. 2020). Furthermore, in some adolescents and young adults, adenomyosis can develop into a large cyst, called cystic adenomyoma (Brosens et al. 2015) (Fig. 6).

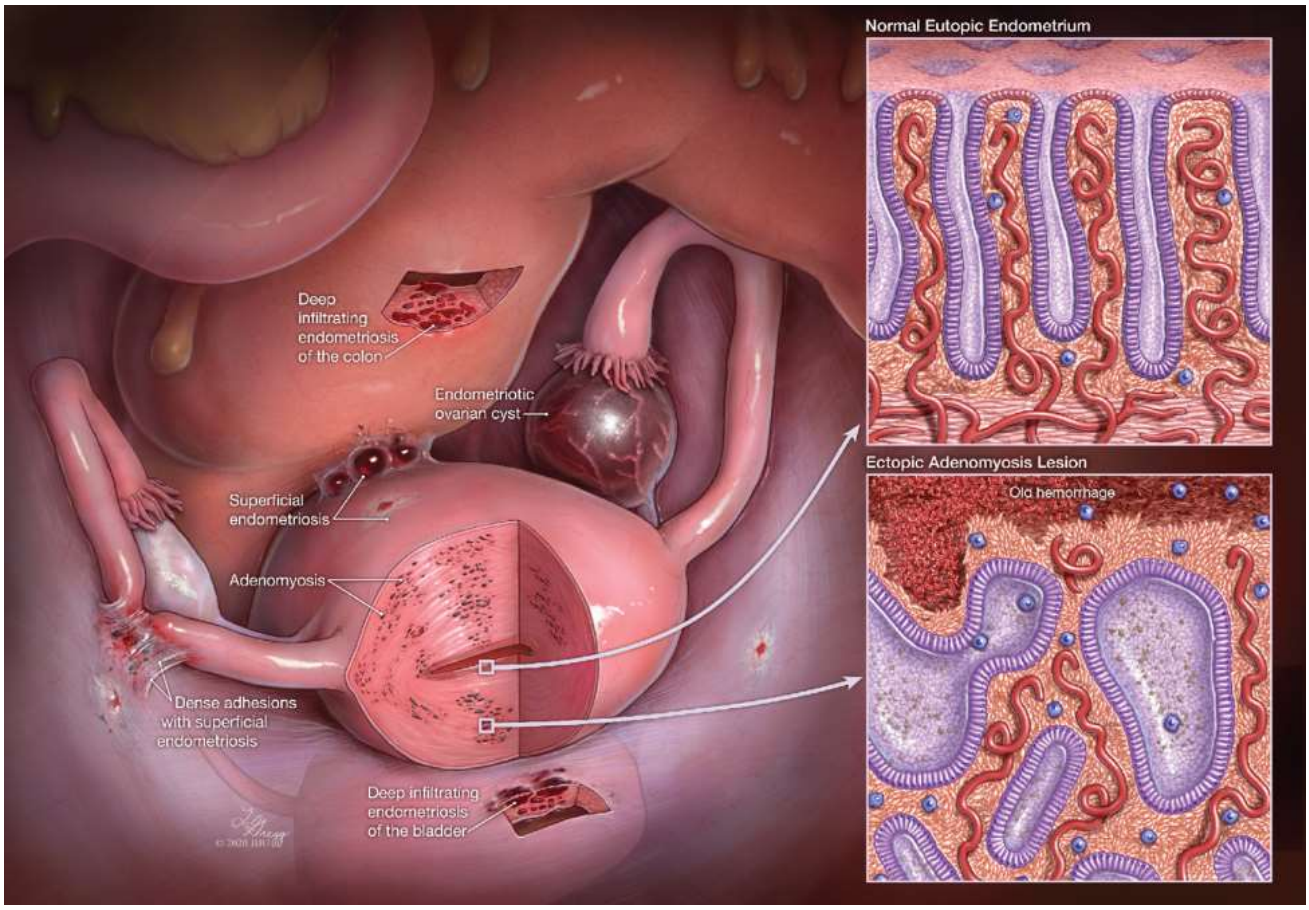

Figure 6 Endometriotic and adenomyotic lesions (Antero et al. 2020)

In general, in women affected by adenomyosis, the uterus may appear slightly enlarged and globular (Shutter 2005) while maintaining its general outline, and only rarely exceeds the size of a 12-week pregnant uterus (Bird, McElin, and Manalo-Estrella 1972). In gross appearance, adenomyosis, unlike leiomyoma, does not have a well-defined boundary. Adenomyotic foci may appear indistinct or as a white-grey mass with areas of brow-staining, secondary to hemolyzed blood and hemosiderin deposits (Azziz 1989). Adenomyomas, on the other hand, are composed of smooth muscle surrounding the endometrial glands and the stroma. The main symptoms of women affected by adenomyosis are abnormal uterine bleeding (AUB), dysmenorrhea and infertility but one third of them are asymptomatic (Peric and Fraser 2006) (Fig. 7). Moreover, a recent meta-analysis has also highlighted a correlation between adenomyosis and the increased risk of spontaneous abortion and pregnancy complications (Nirgianakis et al. 2021) (Fig. 7).

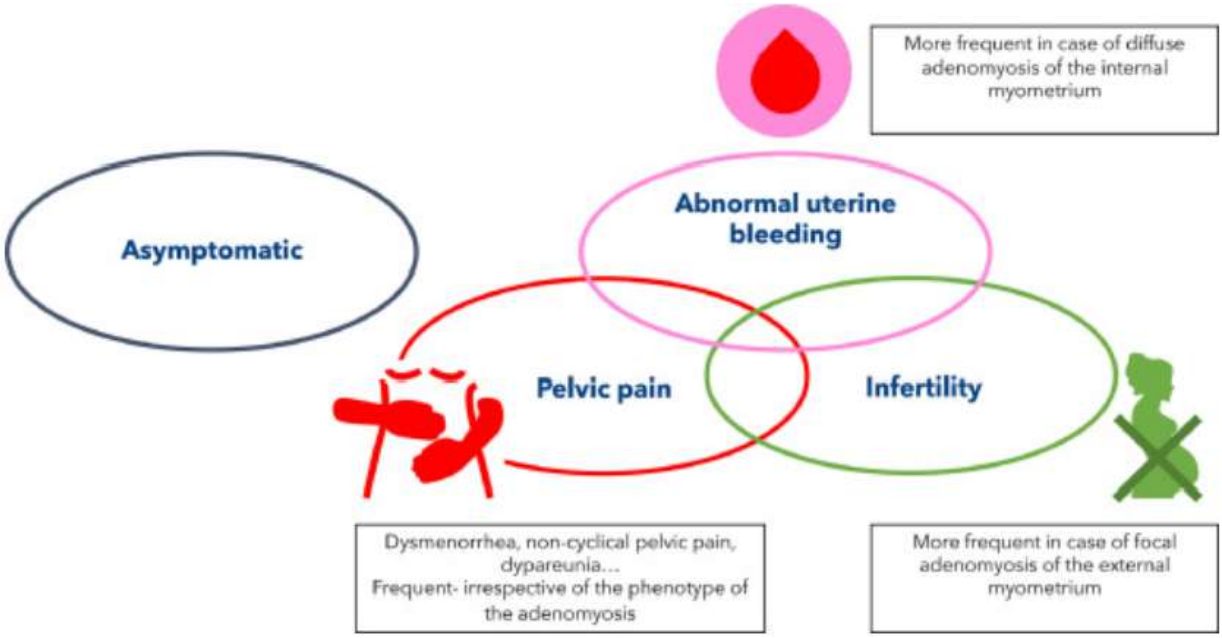

Figure 7 Symptomatology of adenomyosis (Bourdon et al. 2021)

For several years, adenomyosis has remained a histopathological diagnosis made after hysterectomy in perimenopausal women suffering from heavy menstrual bleeding or pelvic pain (Taran et al. 2012). Over the last ten years, adenomyosis has also been identified in young fertile-age women (Pinzauti et al. 2015) thanks to the recent improvements in imaging techniques. However, even though the advancements of diagnostic tools, the awareness of the disease is still poor. Moreover, in some patients, adenomyosis coexists with other gynecological disorders, such as endometriosis and uterine fibroids (G. Leyendecker et al. 2015). According to Kishi et al. and Chapron et al., endometriosis is observed in over 90% of patients presenting adenomyosis. Moreover, the authors demonstrated a difference in the prevalence of endometriosis according to the site of the adenomyosis lesion in the myometrium (Kishi et al. 2012; Chapron et al. 2017). Based on this evidence, some authors hypothesized that adenomyosis and DIE lesions may share a common origin, with the latter being the result of adenomyosis or *vice versa*. In the first scenario, DIE lesions would develop through the extensive proliferation and invasion of adenomyotic lesions in nearby ectopic tissues (G. Leyendecker et al. 2015). On the other hand, it is possible that the regurgitation of menstrual flow in the abdominal pelvic cavity, often attributed to the insurgence of endometriosis, may lead to the development of adenomyosis (Chapron et al. 2017).

A clear diagnosis of adenomyosis is based on the presence of ectopic endometrial tissue in the myometrium by pathological analysis. In the presence of advanced adenomyosis, the uterus usually appears enlarged, or even globular in the most extreme forms. This is mainly due to hyperplasia/hypertrophy of the smooth muscle surrounding the sites of adenomyosis.

Even though the origin of adenomyotic lesions remains unknown, three major theories have been proposed over the years: (I) invagination of basalis endometrium into the myometrium as a result of activation of TIAR mechanism; (II) metaplasia of displaced embryonic pluripotent Müllerian remnants or differentiation of adult stem/progenitor cells; and (III) "invasion from outside" (Fig. 8).

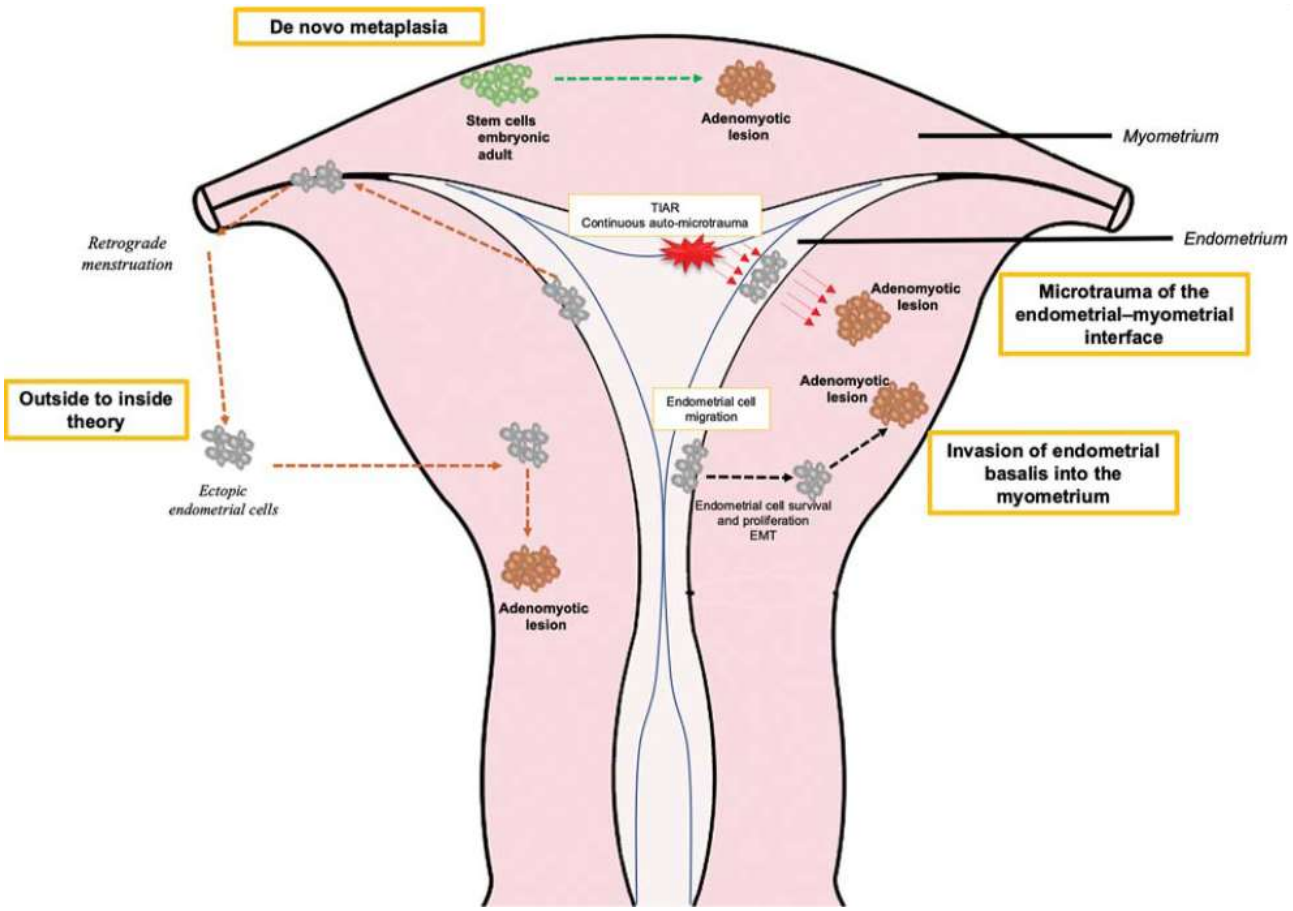

Figure 8 Theories on the Origin of Adenomyosis (Zhai et al. 2020)

According to the invagination theory, the hyperestrogenic environment of the uterus causes chronic contractions of the myometrium, with subsequent injury to the endometrial-myometrial junctional zone (JZ) (G. Leyendecker, Wildt, and Mall 2009). The last one represents a highly specialized hormone-responsive structure located at the endometrial-myometrial interface (Brosens et al. 2010). As proof of tissue microtrauma, levels of anti-smooth muscle antibody–positive and collagen I–positive myofibroblasts are significantly increased in the JZ of women with adenomyosis than in those without (Ibrahim et al. 2017). The TIAR mechanism is then activated in response to the injury and inflammatory cells, such as macrophages, accumulate in an attempt to repair the damage, resulting in chronic inflammation and increased production of estrogen (G. Leyendecker et al. 2015).

As a result, a positive feedback mechanism of increased release of estrogen, wound healing, and auto-traumatization commences, leading to constant disruption of the muscular fibers in the myometrial wall and consequently increased invagination of the endometrial basal layer into the myometrium (Gerhard Leyendecker and Wildt 2011; García-Solares et al. 2018) (Fig. 8). Recent studies have shown that the treatment of endometrial stromal cell cultures from adenomyosis patients with estradiol significantly increased their proliferation rates (Y. Wang et al. 2021) and invasive capacity (Y.-J. Chen et al. 2010). Moreover, it has been suggested that estradiol promotes VEGF expression in epithelial and endothelial cell lines of the endometrium and an increased migration capacity of endothelial cells *in vitro*, while estradiol blockade attenuates these effects (T.-S. Huang et al. 2014), leading the authors, to suppose that this type of interaction is also crucial during the development of human adenomyosis. Further studies have reinforced the hypothesis of invasiveness, highlighting that the adenomyotic glands appear to resemble those of the eutopic endometrium (Maier et al. 2020). In addition, single-cell transcriptomic data revealed a clear resurgence of genes associated with cell motility and cancerous features in adenomyosis (Z. Liu et al. 2021). Moreover, a new theory, named EMID (endometrial-myometrial interface disruption) has been suggested. This theory reviews the TIAR hypothesis, and claims that EMID caused by uterine surgeries could result to a "iatrogenic" adenomyosis later in life (García-Solares et al. 2018; Hao, Liu, and Guo 2020). It was demonstrated that EMID, both mechanically and thermally produced, can lead to adenomyosis in mice, and the likelihood of inducing adenomyosis appears to depend on the severity of the EMID (Hao, Liu, and Guo 2020). The EMID theory includes EMT, recruitment of bone-marrow-derived stem cells, and improved survival of dispersed and displaced endometrial cells through iatrogenic procedures, in addition to hypoxia at the injury site. Although the invasion hypothesis is the most broadly accepted theory in the scientific community, adenomyotic lesions may also result *de novo* from metaplasia of displaced embryonic pluripotent Müllerian remnants or differentiation of adult endometrial stem cells. The Mullerian ducts are essential embryological structures, composed of surface epithelium and urogenital ridge mesenchyme, that develop into the female uterine tract during fetal life (Sobel, Zhu, and Imperato-McGinley 2004). It was suggested that metaplastic changes of intramyometrial embryonic pluripotent Müllerian remnants in the adult uterine wall may potentially lead to development of *de novo* ectopic endometrial tissue within the myometrial wall, creating adenomyotic lesions (García-Solares et al. 2018) (Fig. 8). The Müllerian metaplasia theory is supported by Signorile et al., who found displaced endometrial tissue in fetuses in different ectopic locations, including the posterior wall of the uterus (Signorile et al. 2009). Moreover rare cases reports of confirmed adenomyosis in women with Rokitansky-Küster-Hauser syndrome (absence of functional endometrium) allude to the existence of a different pathogenic invagination mechanism (Chun, Kim,

and Ji 2013). On the other hand, it is possible that development of adenomyotic lesions is an outcome of differentiation of adult stem/progenitor cells residing in the uterus. These stem cells are supposed to reside within cell niches in the endometrium basalis to ensure the regeneration and replacement of cells in the healthy endometrium. However, the presence of these cells can also contribute to unregulated proliferation that may extend beyond the endometrium (Stratopoulou, Donnez, and Dolmans 2020) (Fig. 8). Finally, to explain the origin of adenomyosis, a theory called "invasion from the outside" has been postulated. According to this hypothesis, adult endometrial cells can be transported into the myometrium due to the phenomenon of retrograde menstruation and the ability of ectopic endometrial cells to migrate and invade the pelvic peritoneum. These cells seem to have the ability to invade pelvic organs as well as uterine walls and to create intra-myometrial endometrial implants (Fig. 8). This theory appears to be supported by the robust association between posterior focal adenomyosis and deep infiltrating endometriosis nodules in the posterior compartment in patients with endometriosis/adenomyosis (Chapron et al. 2017).

1.3.2 Pathogenesis and progression of adenomyosis

The pathogenic mechanisms implicated in adenomyosis need to be fully clarified, but in the last ten years many studies have shown that sex steroid hormone aberrations, inflammation, fibrosis and neuroangiogenesis play an important role (Vannuccini et al. 2017; Carrarelli et al. 2015; 2017) (Fig. 9).

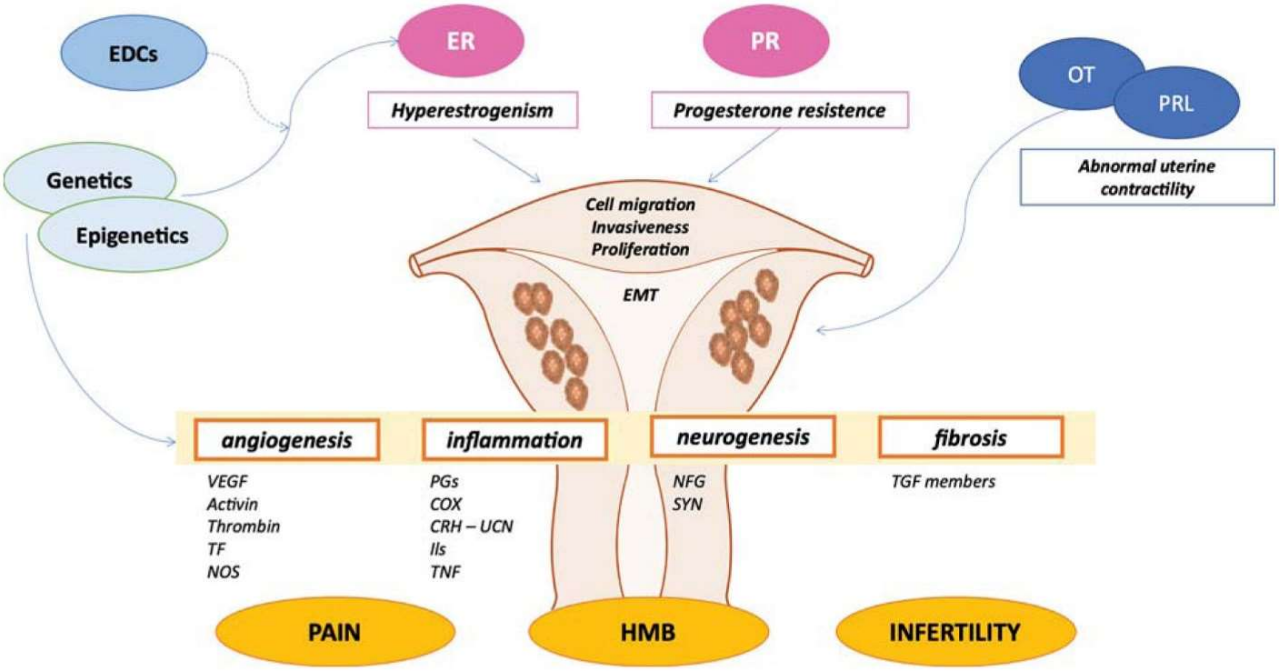

Figure 9 Pathogenesis of adenomyosis (Zhai et al. 2020)

Estrogen and progesterone are the key regulators of a healthy endometrial physiology, necessary for a regular menstrual cycle and to create a suitable environment for embryo implantation. There is a crucial imbalance between estrogen and progesterone signalling during reproductive life in women with adenomyosis (Mehasseb et al. 2011; Rossi et al. 2022). In particular, high local production of estrogens has been demonstrated, with normal peripheral levels of estradiol in adenomyotic lesions as a result of elevated aromatase expression (Kitawaki 2006). Moreover, a genetic study (Tong et al. 2014) found a cytochrome P450 aromatase polymorphism, associated with high local estrogen production in the eutopic endometrium of patients with adenomyosis. Estrogen receptor alpha (ERα) gene polymorphisms are also associated to an enhanced incidence of adenomyosis with increased ER-beta expression in the myometrium of the adenomyotic uterus, contributing to myometrial hyperplasia (Mehasseb et al. 2011). The imbalance between estrogen and progesterone signalling is also determined by a decreased progesterone activity. Indeed, it was observed that women with adenomyosis showed reduced immunoreactivity for isoform B of the PR (PR-B), causing a loss of P effects and strengthening the abnormal endometrial growth (Mehasseb et al. 2011). Recent studies based on next-generation sequencing (NGS) are showing that *KRAS* mutations, a cancer-associated gene, are more likely to be found in patients with concomitant adenomyosis and endometriosis, resulting in inadequate PR expression (Inoue et al. 2019). Mutations that activate *KRAS* induce signalling pathways, which in turn improve cell survival and proliferation and are linked to resistance to progesterone in adenomyosis (Bulun et al. 2021). In conclusion, local endometrial hyperestrogenism and progesterone resistance presumably contribute to increased proliferation of endometrial and myometrial smooth muscle, the EMT process, endometrial angiogenesis, and to junctional area microtrauma, playing a central role in the pathogenesis of adenomyosis. A key mechanism in the development of adenomyosis is represented by the excessive proliferation of endometrial cells that escape from programmed cell death. Indeed high proliferative activity has been described in the ectopic endometrium of patients affected by adenomyosis compared to controls, as demonstrated by immunohistochemical analysis for nuclear antigen of proliferating cells (Xishi Liu et al. 2016a). A recent study also demonstrated the upregulation of mRNA and B-cell lymphoma protein 2 (Bcl-2) in the eutopic endometrium from patients with adenomyosis, indicative of resistance to apoptosis (J. Li et al. 2019). At the onset of adenomyosis, invasion of the myometrium by endometrial tissue is essential for establishment of adenomyosis. It has been suggested that EMT is a key event that enhances the migratory and invasive ability of adenomyotic lesions (Y.-J. Chen et al. 2010). EMT mechanisms can be activated by ER expression, PR downregulation and platelet activation, in combination with chronic hyperperistaltic activity (Rossi et al. 2022). EMT was first described in adenomyosis in a 2010 study, in which the authors observed downregulation of E-

cadherin in association with upregulation of vimentin in the epithelial cells of adenomyotic lesions (Y.-J. Chen et al. 2010). Furthermore, subsequent *in vitro* experiments demonstrated that gene expression modifications, along with the acquisition of cell migration ability, were estrogen-dependent, as blocking estrogen signalling completely eliminated these effects (Y.-J. Chen et al. 2010). Since then, several factors have been proposed as potential regulators of the EMT process in adenomyosis (Khan et al. 2015; Xishi Liu et al. 2016a; Zheng et al. 2018; Hu et al. 2020). For example, recent studies have suggested that focal adhesion kinase (FAK) expression is enhanced in the eutopic endometrium of women with adenomyosis than in controls, and that FAK levels are positively correlated with dysmenorrhea and pelvic pain (Mu et al. 2015). Annexin A2 (ANXA2) was shown to be significantly increased in the ectopic endometrium rather than in the eutopic endometrium of women with adenomyosis. ANXA2 overexpression may be induced by estrogen and it is strongly related to EMT markers and to the severity of dysmenorrhea in patients (S. Zhou et al. 2012). Furthermore, a high Talin 1 mRNA expression was found in women with adenomyosis. Talin 1 through the activation of the canonical WNT / β-catenin pathway, plays an important role in inducing both EMT and increased migration and invasiveness in adenomyotic cells (Y.-Y. Wang et al. 2021). Fibrosis represents another important mechanism implicated in the pathogenesis of adenomyosis (Rossi et al. 2022), and it can be induced by several factors. TGF-β family signalling can regulate metaplasia and smooth muscle fibrosis by acting through a Smad2 / 3-dependent signalling pathway (Cheong, Lai, and Wu 2019). Furthermore, myostatin and activin A regulate the growth of myometrial cells and promote muscle development. An increased expression of these molecules has also been found in the eutopic endometrium of patients with adenomyosis supporting their involvement in the disease (Carrarelli et al. 2015). Another important feature of adenomyosis is chronic inflammation represented by abundant inflammatory mediators in adenomyotic lesions and in peritoneal fluid (S.-W. Guo 2020). Both eutopic endometrium and adenomyotic nodules express elevated levels of IL-1β and corticotropin-releasing hormone (CRH), key inflammatory mediators in the progression of endometriosis (Carrarelli et al. 2017). An increased expression of the IL-18 receptor was also reported in women with adenomyosis compared to controls, highlighting a role of chronic inflammation in the development of adenomyotic lesions (H.-Y. Huang et al. 2010). Moreover, enhanced levels of NF-κB and p65 subunit has been demonstrated in the eutopic endometrium and adenomyotic lesions of patients with adenomyosis (B. Li et al. 2013). Finally, activation of the toll-like receptor (TLR4) induced by lipopolysaccharides, promoted the proliferation and invasion of stromal cells, and amplified a local inflammatory response through several growth factors, leading to the establishment of adenomyosis (J. Guo et al. 2016). This imbalance between pro-inflammatory and anti-inflammatory signals, linked to platelet activation, may consequently

favor the migration of endometrial cells into the myometrium and the activation of EMT. An abnormal and enhanced vascularization has been observed in adenomyosis (Harmsen et al. 2019), and confirmed by the increased density of microvessels in both the ectopic and eutopic endometrium (Schindl et al. 2001). It has been shown that VEGF is overexpressed in patients with adenomyosis (Filippi et al. 2016; Yalaza et al. 2020). In particular, the expression of VEGF appears to be caused by an enhanced expression of the hypoxia inducible factor (HIF-1) action in response to hypoxic stimuli (Goteri et al. 2009). Two other growth factors mainly involved in neoangiogenesis are two members of the TGF-β family named Follistatin and Activin A. The latter in particular, increases the production of VEGF by endometrial stromal cells, modifying the vascularization and leading to the generation of new capillaries (Carrarelli et al. 2016; Bulun et al. 2021). Hypoxia can be characteristic of adenomyosis, resulting from injured JZ, with subsequent lesion to vessels and loss of blood perfusion (Goteri et al. 2009). In fact, aberrant expression of HIF-1α has been observed in adenomyosis and may cause adenomyosis progression and heavy menstrual bleeding (Maybin et al. 2018). Finally, neuroangiogenesis plays a central role in the pathogenesis and pathophysiology of adenomyosis, as an important contributor to pain (Vannuccini et al. 2017). It was demonstrated that adenomyotic lesions express high levels of neurogenic factors, such as nerve growth factors (NGF), synaptophysin (SYN), and microtubule-associated protein 2 (MAP2), compared with controls. NGF production can be stimulated by hyperestrogenism itself and it may induce mast cells to grow and degranulate, producing inflammatory mediators. This results to the production of peripheral nociceptors, enhancing pain perception (Goteri et al. 2009). Furthermore, the inflammatory mediators, IL-1 and TNF-B, increase NGF levels, supporting a link between the inflammatory and neurogenic pathways (Luddi et al. 2019).

1.3.3 Treatments

Adenomyosis is a uterine disorder that affects women of different ages with several symptoms. The management of these patients is still debated. Few clinical studies focusing on the medical or surgical treatment of adenomyosis have been conducted. Vannuccini and colleagues (Vannuccini et al. 2018), clarified in an exhaustive and in-depth way, that currently no drug is specifically labeled for adenomyosis and there are no particular guidelines to follow for better management of the disease. The rationale for using medical therapy is based on the pathogenic mechanisms of adenomyosis. Numerous non-hormonal (non-steroidal anti-inflammatory drugs) and hormonal (oral contraceptives, progestins, gonadotropin-releasing hormone analogues) treatments are used off-label to control pain and bleeding symptoms. Gonadotropin-releasing hormone analogs are recommended before fertility

treatments to increase the chances of pregnancy in infertile women suffering from adenomyosis. 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. Finally, the intrauterine device releasing levonorgestrel is highly effective in the resolution of AUB and the reduction of uterine volume in a long-term management plan. Based on new discoveries on the pathogenetic mechanisms of adenomyosis, new drugs are being developed for its treatment, like as selective progesterone receptor modulators, aromatase inhibitors, valproic acid, and anti-platelets therapy.

1.4 UTERINE FIBROIDS

1.4.1 Introduction

Uterine fibroids (also known as leiomyomas and myomas) are benign monoclonal tumours of the myometrium, representing the most common neoplasms in women worldwide. Uterine fibroids were originally known as the "uterine stone". In the second century, they were named scleromas. The term fibroid was first introduced in the 1860s (Q. Yang et al. 2021). They made up of smooth muscle cells and fibroblasts and characterized by an abundant ECM (E. A. Stewart 2001). Fibroids appear to develop in response to the menstrual cyclicality of gonadal steroids (mainly estrogen and progesterone). Due to their hormonally reactive nature, fibroids primarily affect women during the reproductive years, are very rare before menarche and typically regress after menopause (Marsh and Bulun 2006). The composition and size of uterine fibroids are heterogeneous among women and within the same individual, and their numbers vary among individuals (Jayes et al. 2019). Despite their benign nature, fibroids are a major source of morbidity for women of reproductive age. They can cause heavy or prolonged menstrual bleeding, which in turn often leads to the development of iron deficiency and anaemia. In addition, they can also lead to urinary (such as frequent urination, nocturia or urinary retention) and gastrointestinal symptoms (such as diarrhoea or constipation), or they can also be associated with infertility and poor obstetric outcomes. However, up to 70% of women may be asymptomatic despite having large fibroids. The prevalence of fibroids varies by study and country (4.5% -68.6%), type of investigation, method of diagnosis, and racial/ethnic demographics of the population studied (Pavone et al. 2018). The main risk factor of fibroids include race: it has been shown that fibroids are more common, numerous and larger in African women than in white or Asian women (Marsh et al. 2014). Other risk factors are represented by genetic alterations, obesity, nulliparity, hypertension, late menopause, early menarche, family history of fibroids and advanced age (Fig. 10).

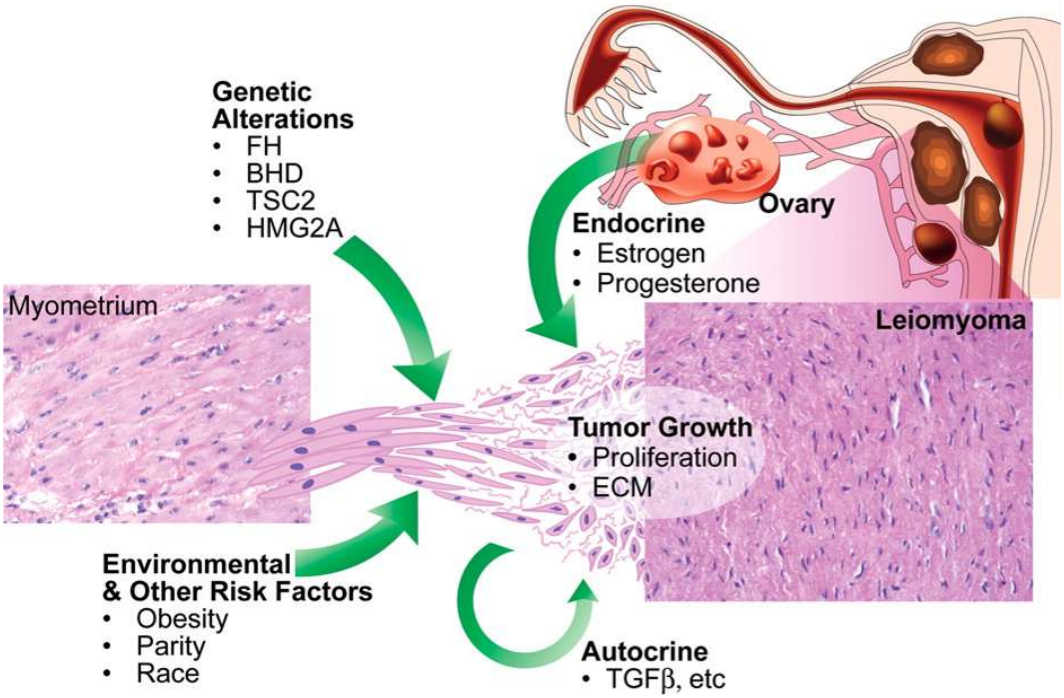

Figure 10 Etiology of uterine fibroids (Walker and Stewart 2005)

The International Federation of Gynecology and Obstetrics (FIGO) has implemented a classification system of the causes of AUB in women of child-bearing age, based on data obtained from imaging. The system uses an 8-point numerical system to characterize the location of fibroids relative to the endometrium (submucosal surface) and the serosal surface, with low numbers indicating a central location (Mg et al. 2011) (Fig. 11).

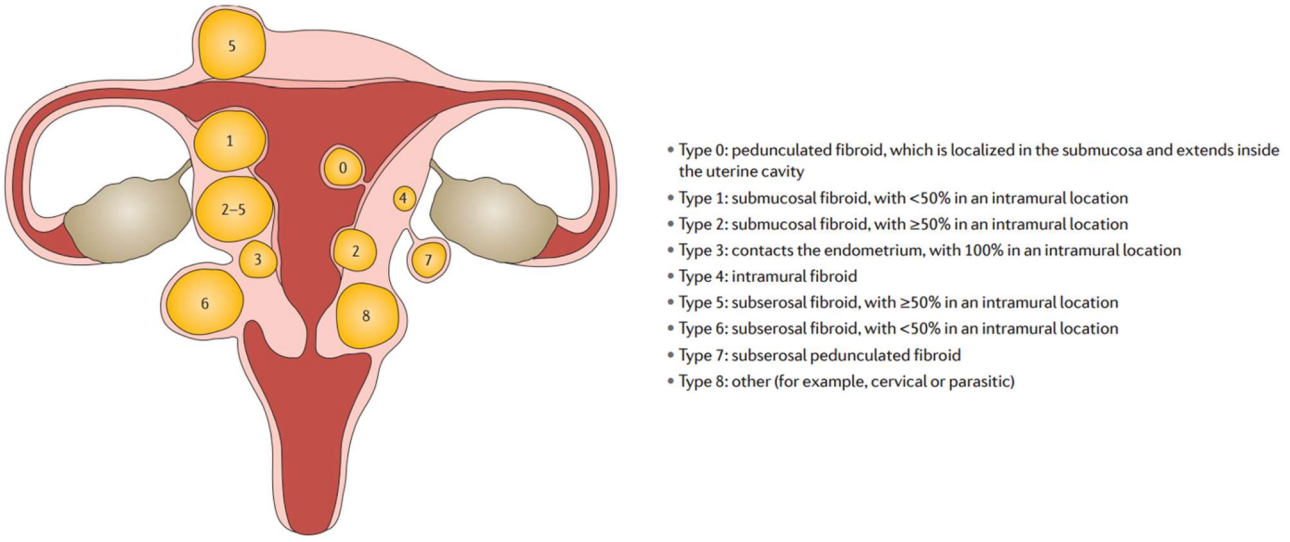

Figure 11 Classification of uterine fibroids (Elizabeth A. Stewart et al. 2016)

Instead, from a histological point of view, uterine fibroids can be subcategorized into five different types: (I) the "usual leiomyoma", (II) the "cellular leiomyoma" that displays increased cellularity (Rosai 2011), (III) the "lipoleiomyoma" which exhibits numerous adipocytes (Avritscher et al. 2001), (IV) the "apoplectic leiomyoma" that shows stellate zones of recent haemorrhage (Myles and Hart 1985) and finally the rare, "bizarre leiomyoma" (Toledo and Oliva 2008; Ciarmela et al. 2012). Among them, the usual leiomyoma is the most frequently variant with an incidence equal to approximately 94% and it is what is commonly referred to as a leiomyoma unless otherwise specified.

1.4.2 Pathogenesis and progression of uterine fibroids

The cellular origin of uterine fibroids is still unknown. Several findings support the idea that each fibroid arises from the transformation of a single somatic stem cell of the myometrium under the influence of ovarian hormones (Mas et al. 2012). The myometrial stem cells express low-to-absent levels of receptors for estrogen and progesterone, but require these steroids for growth, suggesting that the development of clinical disease is dependent on a paracrine mechanism and a multistep process from transformation to the fibroid progenitor through to growth acceleration (Ono et al. 2012). This paracrine interaction with the surrounding cells supports the self-renewal of fibroid stem cells, and it is mediated by the WNT–β-catenin pathway (Ono et al. 2013). This pathway can stimulate the expression of TGFβ3, which induces fibronectin (an ECM protein) expression and cell proliferation in preclinical fibroids more than in the myometrium (Tanwar et al. 2009). Uterine fibroids are considered estrogen-dependent tumors, based on their association with reproductive age (Ishikawa et al. 2009). Several aberrations in estrogen and progesterone signalling pathways are involved in uterine fibroid pathobiology (Borahay et al. 2017). Many studies have demonstrated an enhanced proliferation of uterine fibroid cells *in vitro* after exposure to both estradiol and progesterone (K et al. 1991; H et al. 1999; Cermik, Arici, and Taylor 2002). Recently, it has been showed a primary role of estrogen and ERαto induced progesterone receptor expression and to allowed progesterone receptor ligands to act on their target cells (Bulun et al. 2015). An uterine fibroid xenograft animal model showed that steroids are necessary for tumor growth (Ishikawa et al. 2010), supported by selective progesterone receptor modulators (SPRMs) (Ali and Al-Hendy 2017). Polymorphisms in the estrogen and progesterone receptors and elements of their signalling pathways are also implicated in fibroid biology (Hsieh et al. 2003). Moreover, there is evidence that enhanced local aromatase expression, which converts circulating precursors into estrogens, has a critical role in fibroid development, especially in black women (Ishikawa et al. 2009). Interestingly β-catenin inhibitors and histone deacetylase (HDAC) inhibitors (HDACi) have shown antiproliferative effects

on uterine fibroma cells, thus representing a promising therapy for uterine fibroids (Ulin et al. 2020). In addition to oestrogen, progesterone and WNT–β-catenin signalling, also the influence of specific driver mutations gives a crucial contribution to the stem cell differentiation into a preclinical fibroid. Hierarchical gene clustering has discovered four key pathogenetic subgroups of fibroids, based on somatic mutations or chromosomal alterations in key genes; the mediator complex subunit 12 (*MED12*) group, the high mobility group AT-hook 2 (*HMGA2*) group, the fumarate hydratase (*FH*) group and a rare group correlated with deletion of collagen type IV α5 (*COL4A5*) and *COL4A6* (Mehine et al. 2013; 2014). *MED12* is a component of the mediator complex, which regulates transcription (Borggrefe and Yue 2011). *MED12* mutations determinates a penetrant phenotype which seems to affect the interaction between *MED12* and *cyclin C*, that controls β-catenin transcriptional activity (Mehine et al. 2014; Turunen et al. 2014). The expression of WNT4, an activator of β-catenin, is significantly enhanced in fibroids with *MED12* mutations as compared with those without these mutations (Markowski et al. 2012). Moreover, it was showed that *MED12* deficiency activates the TGF-β pathway, leading to drug resistance and fibroid-cell proliferation (S. Huang et al. 2012). These observations suggest a mechanism implicating *MED12* mutations, WNT–β-catenin activation, and hyperactive TGF-β signalling that supports stem-cell renewal, cell proliferation, and fibrosis in uterine fibroids. In leiomyomas, the dysregulation of *HMGA2*, a transcription-regulating factor (Hodge et al. 2009) might be associated with fibroid growth by the enhanced expression of CDKN2A, which encodes ARF (p14). It was demonstrated that intact ARF (p14) preserves senescence in fibroids (Markowski et al. 2011). Interestingly, uterine fibroids are deficient in the Let-7 miRNA which targets and suppresses *HMGA2* (Peng et al. 2008). Therefore, alterations in the Let7–Via HMGA2 – p14ARF in fibroid stem cells can promote self-renewal and offset senescence. Inactivating mutations of the *FH* enzyme, which plays a key role in the Krebs cycle, have been found in uterine fibroids. Mutations in *FH* result in a change in cellular metabolism, activating hypoxia signalling (Ono et al. 2013). Finally, deletions of the collagen genes *COL4A5* and *COL4A6* are also correlated with a familial syndrome, known as diffuse leiomyomatosis with Alport syndrome and rarely with non-syndromic fibroids (Mehine et al. 2013; 2014).

Uterine fibroids are a fibrotic disorder (Leppert, Catherino, and Segars 2006; Malik et al. 2010) that showed exaggerated and continuous wound healing triggered by tissue injury and characterized by excessive production of ECM (Rafique, Segars, and Leppert 2017) (Fig.12).

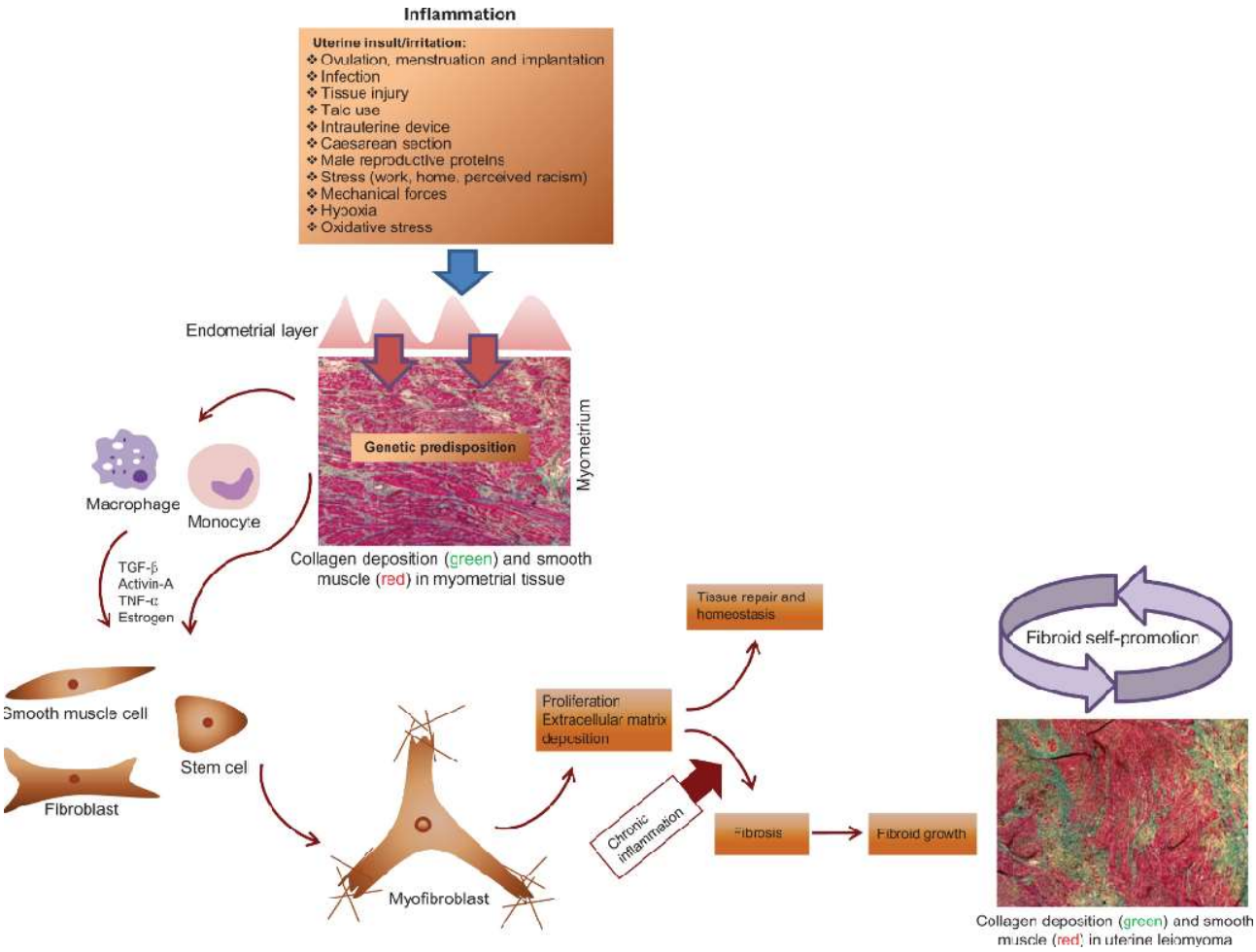

Figure 12 Fibrosis development in uterine leiomyoma (Islam et al. 2018)

In particular, several studies have shown that uterine fibroids contain approximately 50 % more ECM, comprised of collagens, fibronectin and versican than the surrounding myometrium (Islam et al. 2018). This increase creates a milieu of enhanced mechanical stress which is transmitted to the cells by a process named mechanotransduction, or mechanical signalling. The cells respond to these mechanical stimuli by activating downstream signalling pathway which play an important role in the pathogenesis of uterine fibroids (Rafique, Segars, and Leppert 2017). The accumulation of ECM is a key event in the production of the rigid structure of leiomyoma, and ECM stiffness is considered a cause of abnormal bleeding and pelvic pain. Fibrotic responses occur upon recruitment of inflammatory cells to the site of injury and the activation of collagen producing fibroblasts (Kisseleva and Brenner 2008). These activated fibroblasts, also called myofibroblasts, regulate connective tissue remodelling by ECM synthesis/degradation and contraction of the surrounding tissue (Hinz et al. 2012). In a recent study, it was found α-smooth muscle actin (α-SMA) positive and desmin negative cells as well as a significant amount of collagen in leiomyoma tissue, suggesting the presence of myofibroblasts and their role in the ECM deposition (Protic et al. 2016). It is well known that during

women's reproductive age, events such as ovulation, menstruation and implantation may create physiological lesions in the uterus. Furthermore, mechanical forces, hypoxia and oxidative stress can contribute to generate a chronic inflammatory state (Wegienka 2012; Santulli et al. 2013; Fletcher et al. 2013; Leppert, Jayes, and Segars 2014). During this pathological condition, myofibroblasts continuously and excessively produce ECM leading to the development of fibrotic tissues. For this reason Leppert and coworkers proposed a model of uterine fibroids evolution based on an aberrant response to tissue repair, resulting in disordered healing, myofibroblast transformation, myofibroblast failure to undergo apoptosis and formation of an altered ECM (Leppert, Catherino, and Segars 2006). Growth of uterine fibroids is driven by several growth factors (Ciarmela, Islam, et al. 2011). Among them, the TGF-β has a critical role in contributing to myofibroblast transformation and in the progression of the fibrosis (Fallowfield et al. 2007). Indeed, it was demonstrated that uterine fibroids overexpress TGF-β receptors compared to normal myometrium. Moreover, downstream targets of TGF-β signalling, such as tissue matrix metalloprotease inhibitor and plasminogen activator inhibitor that favourite ECM production, are also enhanced in uterine fibroids. Activin A, another member of the TGF-β family, is a key immuno-regulator produced by macrophages (Sierra-Filardi et al. 2011) and is responsible for myofibroblast transformation in many tissues such as liver, lung and heart (Werner and Alzheimer 2006). In a recent study conducted by Islam et al (Islam, Catherino, et al. 2014), it was demonstrated that activin A has also a direct pro-fibrotic effect on primary leiomyoma cells by inducing the expression of ECM proteins. These results suggest a critical role of activin-A in the fibrogenesis of uterine fibroids, as also supported by the evidence that ulipristal acetate, a hormonal drug used to treat fibroids, reduces activin A expression *in vitro* (Ciarmela et al. 2014).

1.4.3 Treatments

The available treatments for uterine fibroids include medical therapies, interventional radiology, and surgical procedures (Giuliani, As-Sanie, and Marsh 2020). Much international obstetrics and gynaecology societies recommend a step-by-step approach to treat uterine fibroids, starting with pharmacological and minimally invasive treatments before resorting to surgery. Clinical innovations are emerging in the use of progesterone receptor modulators as medical therapy. However, despite these advances, hysterectomy remains the primary treatment of choice for women with symptomatic fibroids.

1.5 SPHINGOLIPIDS

1.5.1 Introduction

Sphingolipids were first isolated from the brain in the late 19th century by Thudicum, who introduced the name "sphingosine" in honour of the Greek mythical creature the Sphinx, in deference to "many enigmas which is presented to the inquisitor" (‘A Treatise on the Chemical Constitution of the Brain: Based Throughout upon Original Researches’ 1884). Sphingolipids are amphipathic molecules, ubiquitously present in eukaryotic cells, whose hydrophobic region consists of a sphingoid skeleton, usually, sphingosine (Sph), to which a fatty acid is linked to carbon in position 2, *via* carbamide bond. The hydrophilic region can be represented according to the sphingolipid, by a phosphate group linked to C-1 (sphingosine 1-P, ceramide 1-P), phosphorylcholine in the sphingomyelin (SM), or by sugars in the glycosphingolipids (GSL). Sphingolipids are distinguished based on the type of carbonaceous skeleton of the fatty acid bound to C-2 (Bartke and Hannun, 2009): more than 20 species of fatty acids, which differ in length, degree of saturation, and degree of hydroxylation can be related to the sphinx skeleton.

Sphingolipids are a class of complex lipids, which have long been known for their essential role in the structural organization of biological membranes. However, since the 1980s, their function has also proved crucial in many other physiological processes, such as proliferation, motility and cell survival, inflammation, fibrosis, and death, and for these reasons they have earned the definition of bioactive lipids. The concept of bioactive lipids has evolved over the past several decades, from studies on inositol phospholipids in the 1950s, to studies on prostaglandins in the 1960s, and the discovery of the bioactivity of diacylglycerol (DAG) in the 1980s. Bioactive lipids are functionally defined as lipid species whose levels react (acute and/or tonic) to the action of specific stimuli. These lipids then control certain downstream effectors and targets. The main bioactive sphingolipids that have received the most attention are ceramide (Cer), Sph, and sphingosine 1-phosphate (S1P). Additional evidence also implicates ceramide-1-phosphate (C1P), glucosylceramide (GluCer), galactosylceramide, and some of the gangliosides as candidate bioactive lipids (Hannun and Obeid 2008).

1.5.2 Sphingolipids metabolism

The sphingolipid metabolic pathway displays an elaborate network of reactions. It is characterized by a unique metabolic entry point, represented by the enzyme serine palmitoyl transferase (SPT), which forms the first sphingolipid in the *de novo* pathway; and a unique exit point, represented by S1P lyase

(SPL), which breaks down S1P into non-sphingolipid molecules. The multiple intermediary metabolic steps constitute a highly complex network which links the metabolism of many sphingolipids. In this pathway, Cer can be considered a metabolic hub thanks to its central position in sphingolipid biosynthesis and catabolism (Fig. 13).

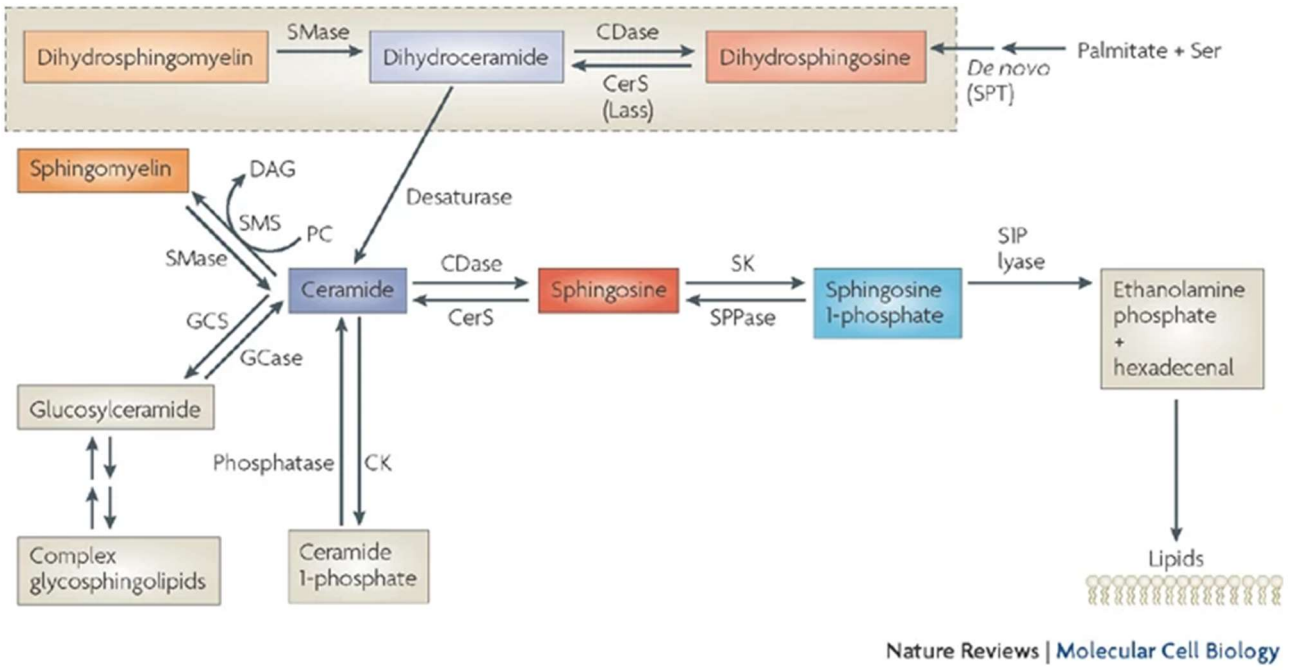

Figure 13 Sphingolipids metabolism (Hannun and Obeid 2008)

Cer can be produced in two distinct ways. First, it can be synthesized through the *de novo* pathway, and second, through the hydrolysis of complex lipids, especially SM. The *de novo* pathway begins in the endoplasmic reticulum (ER), with the condensation of serine and palmitoyl-CoA *via* SPT to generate 3-keto-dihydrosphingosine (Merrill 2002). This enzyme belongs to the α-oxoamine synthase family and is pyridoxal 5′-phosphate dependent (Ikushiro and Hayashi 2011). SPT is a heterodimer consisting of SPTLC1 and SPTLC2 subunits, and each of them, is an integral membrane protein of the ER. Another SPT subunit has recently identified in mammals, SPTLC3, whose expression is restrict to specific tissues such as placenta and trophoblasts, as opposed to SPTLC1 and SPTLC2, that are ubiquitously expressed (Hornemann et al. 2006). 3-Keto-dihydrosphingosine is subsequently reduced to form dihydrosphingosine (sphinganine) by 3-ketosphinganine reductase (3KSR) in a NADPH-dependent manner. Sphinganine is *N*-acylated by dihydro-cer synthases (CerS) to produce dihydro-cer (dhCer). Six mammalian CerS have been cloned. They were called longevity-assurance homolog (LASS1-6)/Cer synthase (CerS1-6), and each isoform shows substrate preference for

specific chain length fatty acyl CoAs, except for LASS3 which has a broad substrate specificity (Pewzner-Jung, Ben-Dor, and Futerman 2006). All six CerS present: a domain called Tram-Lag-CLN8 (TLC), inside which there is a Lag1p motif, a conserved trait of 52 amino acids necessary for enzymatic activity; and a region of 11 amino acid residues located between two transmembrane domains, that is crucial in determining the specificity of acyl-CoA (Tidhar et al. 2018). In mammals, dhCer is then desaturated by dhCer desaturase (DEGS-1) to form Cer (Michel et al. 1997). The transport of the last one to the Golgi occurs either through the action of the Cer transfer protein (CERT) (Hanada et al., 2003), which specifically deliver Cer for SM synthesis or through vesicular transport, which deliver Cer for the synthesis of GluCer, a precursor for the synthesis of complex GSLs. Cer is glycosylated to GluCer at the level of the cytosolic layer of the cis-Golgi membranes. Subsequently, the GluCer must be transported to the trans compartment of the Golgi and translocated on the luminal leaflet. Thefollowing glycosylation reactions leading to the synthesis of complex GSLs. GluCer transfer from cis- to trans-Golgi is mediated by the four-phosphate adaptor protein 2 (FAPP2) (Fig. 14).

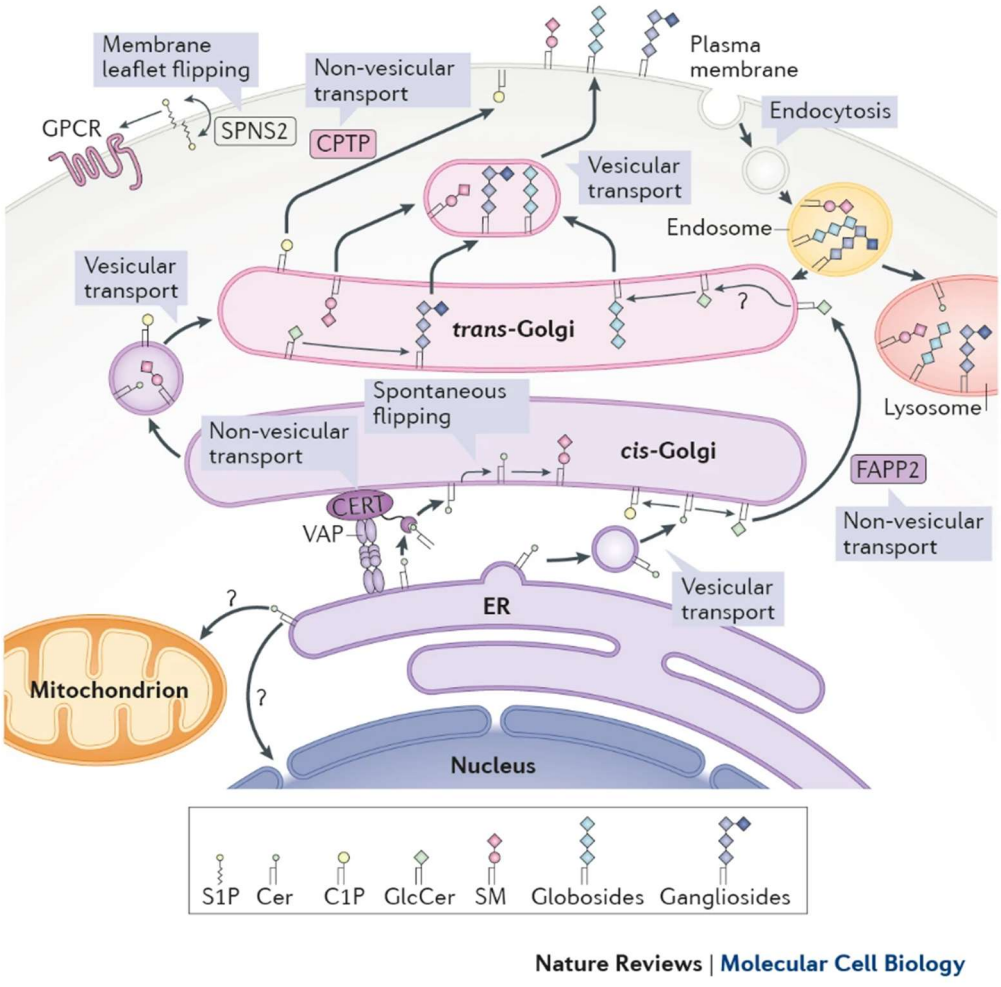

Figure 14 Intracellular compartmentalization and transport of sphingolipids (Hannun and Obeid 2018).

The synthesis of SM is mediated by sphingomyelins synthase (SMSs), which transfers the phosphocholine headgroup from phosphatidylcholine to the Cer, resulting in SM and DAG molecules. There are two isoforms of SMS: the first, SMS1, is an integral membrane protein of Golgi, while the second, SMS2, is predominantly found in the plasma membrane (Huitema et al. 2004). SM, after its translocation to the plasma membrane level, can form part of the lipid pool of the membrane as a structural component or be further metabolized to give rise to bioactive sphingoid molecules. In this degradation process, SM is converted to Cer and phosphorylcholine by the hydrolysis of the phosphodiester bond. This reaction is catalyzed by the enzyme sphingomyelinase (SMase), which breaks down SM to produce Cer and phosphocholine. The SMase-mediated hydrolysis of SM has emerged as a key pathway of stress-induced Cer production and it's stimulated in response to several stimuli. Several SMases are characterized: zinc ion-dependent acid SMases (lysosomal aSMase and secretory acid Smase (aSMase or SMPD1); neutral magnesium ion-dependent SMases (nSMase1, nSMase2, and nSMase3); and alkaline SMase (alk-SMase) (Marchesini and Hannun 2004). SM is converted to Cer by acid SMase. Cer can be deacylated through the action of acid ceramidases (CDases) to yield Sph. The latter can then translocate across the lysosome, where it can be either re-acylated to Cer, or phosphorylated by two sphingosine kinase isoenzymes (SK1 and SK2) to generate S1P. Three types of CDases have been described: acid, neutral and alkaline, and like SMases, they are characterized according to their pH optima and subcellular localization (Cungui Mao and Obeid 2008). S1P represent the terminal product of the metabolism of SLs and can be degraded through two different reactions. The enzyme S1P phosphatase (SPP), of which two isoforms are known (SPP1 and SPP2), catalyzes the reversible degradation of S1P to Sph, by a dephosphorylation reaction. S1P dephosphorylation can also be catalyzed by non-specific phosphatases belonging to the lipid phosphate phosphatase family. The irreversible degradation of S1P, the only exit point from the metabolism of sphingolipids, is instead mediated by SPL which cleaves S1P into ethanolamine-1-phosphate and hexadecenal (palmitaldehyde) (Futerman 2021). The hexadecenal can also be oxidized to palmitate, thus re-entering the lipid metabolism. To complete this already complex metabolic framework, it should be remembered that there is also a lysosomal degradation process of sphingolipids, which mainly affects the GSLs (Mathias, Peña, and Kolesnick 1998). Membrane fragments containing these molecules are internalised in the form of coated vesicles and, passing through the endosomal compartment, reach the lysosomes. Here, through sequential hydrolysis of saccharide residues, catalysed by specific glycohydrolase, Cer is formed which, by CDase acid present at the level of the lysosomes, is converted into Sph. The intermediates of the lysosomal sphingolipid's catabolism, including saccharide residues, fatty acids and Sph, may be further degraded or recycled. Sph generated in lysosomes, for example, may fall in the biosynthetic pathway

of sphingolipids through what is referred to as the "recovery pathway" or salvage pathway (Tettamanti et al. 2003).

1.6 SPHINGOSINE 1-PHOSPHATE

1.6.1 Introduction

S1P is a key cellular mediator. In 1991 was initially discovered the importance of S1P as a regulator of cell growth (H. Zhang et al. 1991). Over the next decade, this sphingolipid was shown to mediate a wide spectrum of other biological processes, including: Ca^{2+} mobilization, survival, cell motility, cytoskeleton remodelling, and immunity (Pyne and Pyne 2002; Sarah Spiegel and Milstien 2003). It is now clear that this relatively simple molecule can affect such an array of several cellular processes because of its ability to function both as an intracellular second messenger, and as a ligand for five specific G protein-coupled receptors (GPCRs), named $S1P_{1-5}$, that are coupled to multiple G proteins and regulate many downstream signalling pathways.

S1P levels in cells, are tightly controlled by the balance between its synthesis, which is catalysed by SKs, and degradation, that is catalysed by specific (SPPs) and aspecific phosphatases and SPL (Fig. 15).

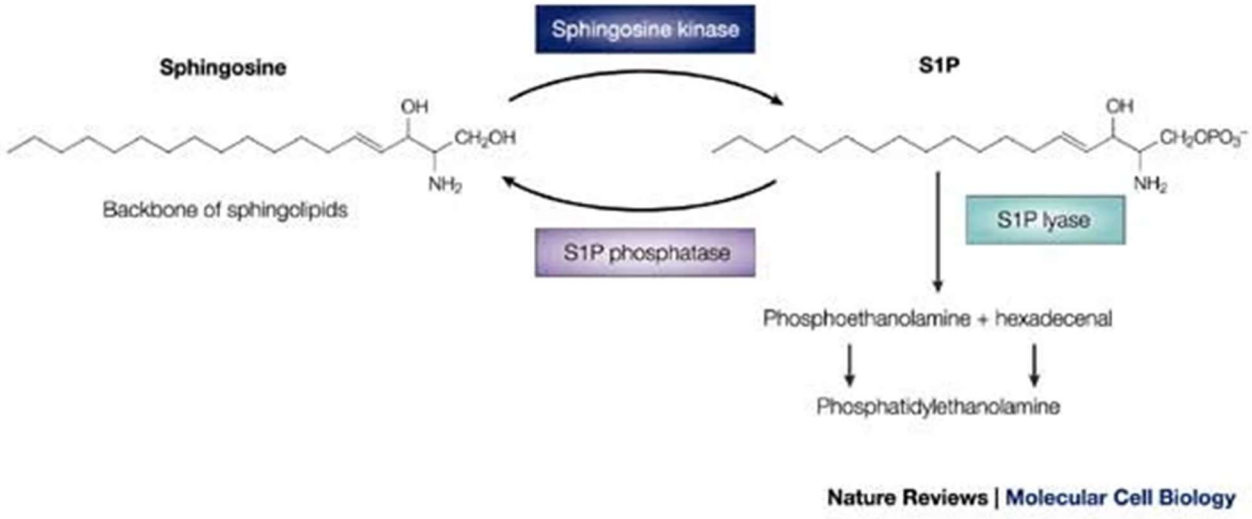

Figure 15 S1P: synthesis and degradation (Sarah Spiegel and Milstien 2003)

This balance between S1P generation and degradation generally results in low cellular levels of S1P (S. Spiegel et al. 1998). However, the levels of this sphingolipid can quickly and transiently increase as a direct result of enhanced SK activity, following stimulation through a varied range of extracellular stimuli, including growth factors and cytokines (Leclercq and Pitson 2006). Furthermore, S1P can be secreted into the extracellular environment *via* many transporters and signals through its receptors (Timothy Hla 2004; Rosen et al. 2009).

The concentration of S1P in plasma ranges from 0.2 to 0.9 µM, whereas in serum, it shifts from 0.4 to 1.1 µM (Caligan et al. 2000; Berdyshev et al. 2005). This S1P gradient is essential for numerous physiologic functions provided by extracellular S1P (Ana Olivera, Allende, and Proia 2013). Within the plasma, most S1P is bound to protein carriers, such as HDL (~60%) and albumin (~30%), with lesser amounts bound to VLDL and LDL (Argraves and Argraves 2007). S1P is bound to HDL *via* the apolipoprotein ApoM, which functions as an S1P chaperone that controls the levels of the bioactive sphingolipid in the blood (Christoffersen et al. 2011) (Fig. 16). Chaperones permit the aqueous solubility of S1P and allow it to be transported as a paracrine and endocrine mediator. Moreover, chaperones such as ApoM may also protect S1P from degradation and facilitate presentation to receptors. High levels of S1P in circulation derived from red blood (rbc) and endothelial cells, which are metabolically oriented toward S1P secretion (Ana Olivera, Allende, and Proia 2013) (Fig. 16). Indeed, rbc are responsible for the production of almost all embryonic S1P (Xiong et al. 2014) and approximately 75% of adult plasma S1P in mice (Pappu et al. 2007; Xiong et al. 2014). The vascular endothelium is another important contributor (Venkataraman et al. 2008) (Fig. 16), whereas platelets are not crucial for plasma S1P concentrations in postnatal homeostatic conditions (Venkataraman et al. 2008; Camerer et al. 2009). Indeed, they may only release S1P during platelet activation and clotting (Fig. 16). The lymphatic endothelium is the principal source of lymph S1P (Pham et al. 2010). By contrast, tissue S1P levels are low, varying between 0.5 and 75 pmol/mg (Edsall and Spiegel 1999; Schwab et al. 2005).

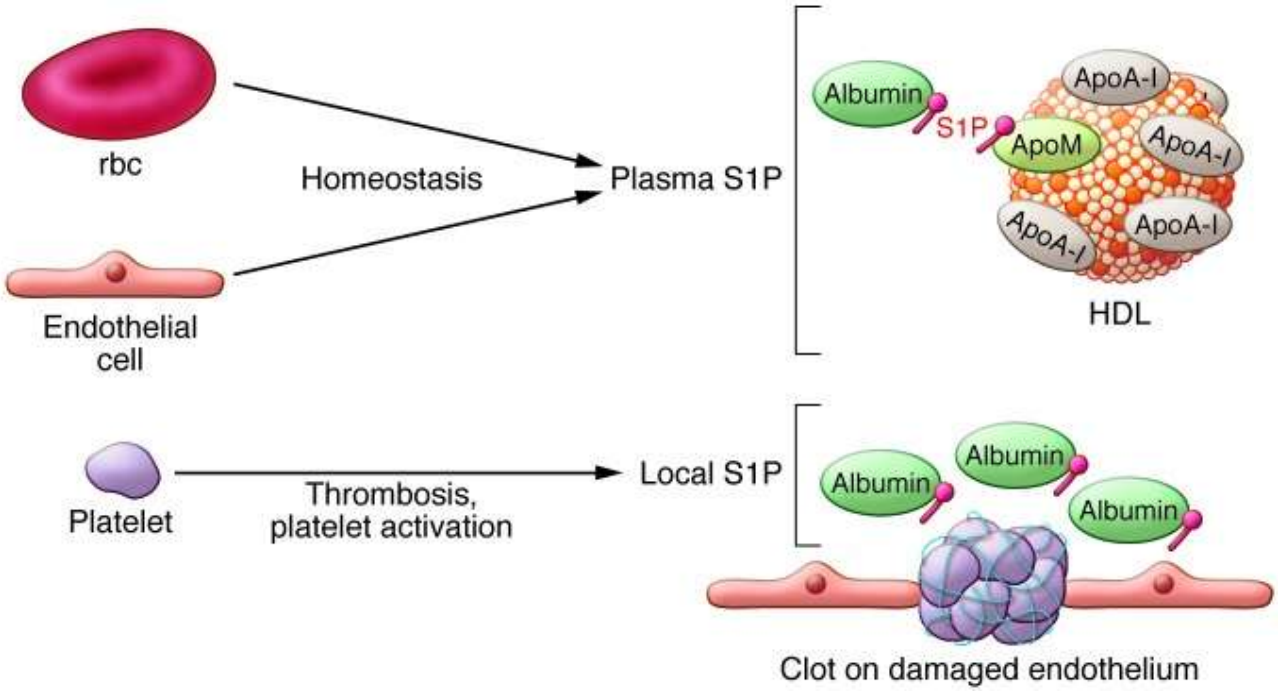

Figure 16 Cellular sources of plasma S1P (Proia and Hla 2015)

Tissue-specific transporters export S1P to establish the extracellular gradients. Original studies on multi-drug resistance, in cancer cells and yeast, identified numerous ATP-binding cassette (ABC) transporters that, besides amphiphilic drugs, mediate the transport of lipids from the inner to the outer leaflet of the plasma membrane (van Meer and Lisman 2002). They are characterized by two transmembrane domains with six membrane-spanning *α*-helices which form a channel for substrate transport through membranes, and by two cytosolic ATP-binding cassettes. According to phylogenetic analysis and alignment of amino acid sequences, the 49 human *ABC* genes can be grouped into seven main subfamilies: *ABCA*-through-*ABCG*. Several studies have drawn attention to the involvement of this family of transporters in the export of S1P from various types of cells (R. H. Kim et al. 2009). For example, S1P can be exported from endothelial cells *via* ABCA1 and ABCC1 (Y.-M. Lee et al. 2007); from mast cells *via* ABCC1 (Mitra et al. 2006); from astrocytes *via* ABCA1 (K. Sato et al. 2007); and from human breast cancer cells *via* ABCC1 and ABCG2 (Takabe et al. 2010). Collectively, these studies suggest that members of the large family of ABC transporters are responsible for the export of S1P in various types of cells. However, in some studies using mice with ABC transporter deficiencies, including animals with a knockout of ABCA1, ABCA7, and ABCC1, S1P levels and related functions have been found unaltered (Y.-M. Lee et al. 2007), indicating the existence of compensatory mechanisms with other transporters. SPNS2 (Spinster homologue 2), is a putative twelve transmembrane domain protein (504 amino acid residues) belonging to the Spinster family, which is part of the major facilitator superfamily (MFS) of non-ATP-dependent organic ion transporters. The transport activity of SPNS2 increases in proportion to the amount of S1P inside the cells, which suggests that it acts as a passive transporter that does not require any energy source (Hisano, 久 et al. 2011). SPNS2 is localized to the plasma membrane and was demonstrated to play a key role in establishing the S1P gradient in the blood, in development and organ homeostasis, and inflammation (Sarah Spiegel et al. 2019). SPNS2 was first identified in a zebrafish screen for cardia bifida (a severe condition that leads to the formation of two beating hearts), and acts in the extraembryonic yolk syncytial layer to export S1P. The latter binds to $S1P_2$ on the cells of the endoderm, and allows directional cardiac progenitor cell migration to the midline and correct cardiac development (Kawahara et al. 2009). This is the first example of S1P extracellular gradients created by a lipid transporter, which is necessary for a developmental event (Kupperman et al. 2000; Osborne et al. 2008). Recently a new member of the same family of transporters as SPNS2 was discovered called Mfsd2b, which is responsible for the releasing of S1P from erythrocytes and platelets. Notably, it was found that its knocking out in mice reduced S1P plasma levels by ~50% (Vu et al. 2017). However, endothelial cells can also release S1P by SPNS2. Endothelial-specific knockout of SPNS2 reduced circulating S1P to the same extent as the global knockout (Fukuhara et al. 2012), and has the

same lymphopenic effects (Nagahashi et al. 2013), demonstrating that SPNS2 secretes S1P from endothelial cells directly to the blood. It can therefore be said that there are at least two sources of circulating S1P, one dependent on Mfsd2b in erythrocytes and platelets, and another dependent on SPNS2 in endothelial cells.

1.6.2 S1P and cell fate decisions

While extracellular S1P has a number of roles mediated *via* ligation to $S1P_{1-5}$, including angiogenesis, cell migration and immune cell function, intracellular S1P has shown to enhance cell survival and proliferation (Strub et al. 2010), possibly through its direct interaction with histone deacetylases 1 and 2 (HDAC1/2) (Hait et al. 2009) and/or TNF receptor-associated factor-2 (TRAF2) (Alvarez et al. 2010). In contrast to S1P, Cer and Sph, have generally been associated with growth arrest and apoptosis (Cuvillier 2002). Relative levels of these lipids therefore appear to determine cell fate. Indeed, a number of studies have shown that targeting the key enzymes involved in the control of this so-called “sphingolipid rheostat” is a strategy for cancer therapy (Shida et al. 2008; Cuvillier et al. 2010; Newton et al. 2015) (Fig. 17). Importantly, the SKs play a central role in the "sphingolipid rheostat" by regulating the relative levels of S1P, Cer, and Sph. Therefore, the study of SKs regulation is fundamental to understand the control of the sphingolipid system.

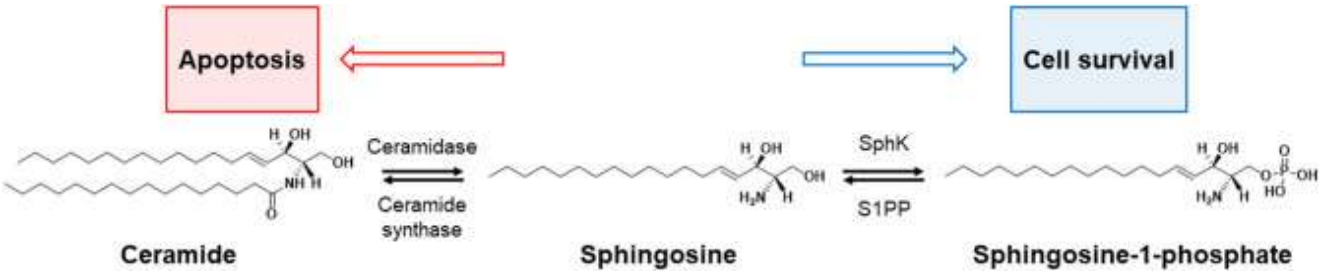

Figure 17 Sphingolipid rheostat (Kroll, Cho, and Kang 2020)

1.6.3 Sphingosine kinases (SKs)

SKs are evolutionarily conserved. They are expressed in humans, mouse, yeast, and plants, with homologous in worms and flies. Two mammalian isozymes, known as SK1 and SK2, have been characterized (H. Liu et al. 2002). Both enzymes share a significant sequence similarity; most of the SK1 sequence aligns within the larger SK2 sequence (with 47% and 43% amino acid sequence

identity for the N- and C-terminal regions of SK1). SK1 and SK2 contain five conserved regions (C1–C5), with the catalytic domain formed within C1–C3 and the ATP binding domain located in the C2 region (Leclercq and Pitson 2006; Pyne, Adams, and Pyne 2016) (Fig. 18).

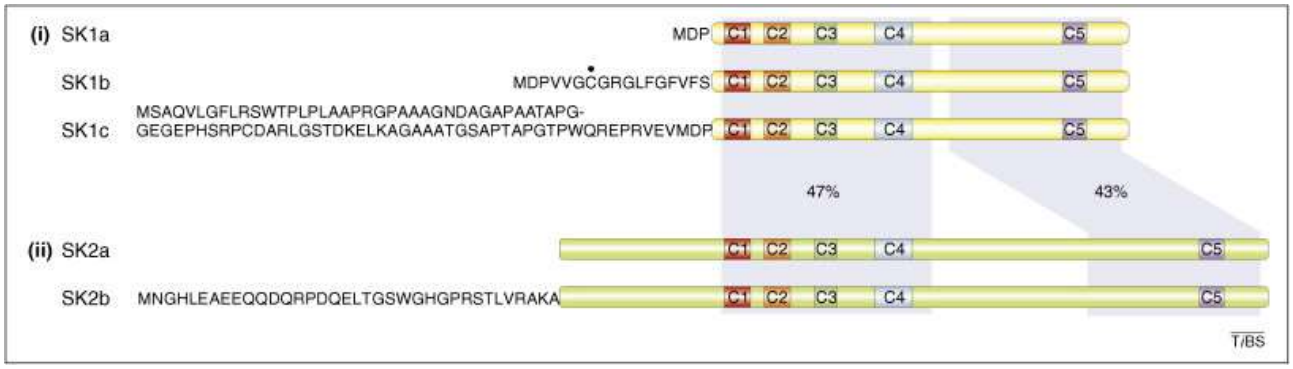

Figure 18 Human SK isoforms (Stuart M. Pitson 2011)

In the human genome, SK1 is localized to chromosome 17 (17q25.2) and SK2 to chromosome 19 (19q13.2). There are three splice isoforms for SK1 (SK1a, -b and -c) that differ only at their N-termini (Fig. 18). Compared to SK1a, SK1b presents 14 additional amino acids, including a Cys which is a putative palmitoylation site, that may explain its enhanced constitutive localization at the plasma membrane. Interestingly, SK1b is more resistive to removal from cells *via* the proteasome (compared with SK1a). SK1c possess a longer N-terminal extension of 86 amino acids. Although detailed analysis of the expression patterns for these SK1 isoforms has not been done, SK1a and -b in human umbilical vein endothelial cells seem to have a similar profusion, which is significantly greater than that of SK1c (Stuart M. Pitson 2011). SK2 has a supplementary region at the N-terminus and within the central part of its sequence that are not present in SK1. Two SK2 isoforms have been reported which appear to be derived from the use of an alternative start codon (Fig. 18). Compared to SK2a (also termed SK2-S), SK2b (or SK2-L) possesses an additional 36 amino acids which have higher catalytic activity, suggesting that the N terminus may contribute to a conformation with enhanced catalytic activity (Stuart M. Pitson 2011). Whereas SK2a induced apoptosis through its putative BH3 domain (H. Liu et al. 2003) and inhibited DNA synthesis both in the absence and presence of serum (Okada et al. 2005), SK2b decreased DNA synthesis only in the absence of serum, suggesting a distinct roles for the two isoforms (Okada et al. 2005). Finally, it was identified another variant of human SK2 that has 761 amino acids and diverge from the previously described SK2 variants by a prolonged N-terminal extension and a modified C terminus (Alemany et al. 2007).

SK1 possess an intrinsic catalytic activity that is not dependent on any eukaryotic post-translational modification, since recombinant human SK1 produced in bacteria is active (S M Pitson et al. 2000).

During normal physiological conditions, SKs are thought to act as housekeeping enzymes that help to regulate the relative levels of Sph, Cer, and S1P in the cell. This low basal activity of SK1, however, can be quickly and temporarily increased upon stimulation by several agonists, including TNF-α (Stuart M. Pitson et al. 2003), IL-1β (Mastrandrea, Sessanna, and Laychock 2005), platelet-derived growth factor (PDGF) (Pébay et al. 2005), VEGF (Shu et al. 2002), epithelial growth factor (EGF), NGF (Rius, Edsall, and Spiegel 1997), and notably S1P itself (Meyer zu Heringdorf et al. 2001). While stimulation by these agonists only results in a modest incremental of cellular SK1 activity (~2-fold), this level of SK1 activation is enough to cause a remarkable increase in cellular and secreted S1P (S. M. Pitson et al. 2000; Stuart M. Pitson et al. 2003). SK1 activation is mediated by phosphorylation on Ser225 by ERK1/2, that results in a 14-fold increase in its catalytic activity but does not change its affinity for either ATP or Sph (Stuart M. Pitson et al. 2003). In most cases, the enhanced SK1 activity by phosphorylation is transitory, due to dephosphorylation at phospho-Ser225 by protein phosphatase 2A (PP2A) (Barr et al. 2008). PP2A isa heterotrimer holoenzyme constituted of three components: structural, catalytic and regulatory subunits (Fig. 19). Successive studies displayed that by interacting directly with the *c*-terminus of SK1, the B′α regulatory subunit of PP2A is necessary for the dephosphorylation of phospho-Ser225 and subsequent deactivation of SK1 (Pitman et al. 2011). While SK1 activation by phosphorylation has been well described, it is, nevertheless, not the only mechanism for post-translational regulation of this enzyme. Many studies have now demonstrated that SK1 activity can be modulated through other mechanisms, such as its interaction with several proteins. A range of SK1-interacting proteins has been identified, with different effects on SK1 regulation. Some activate SK1, others inhibit, while others alter its subcellular location (Fig. 19).

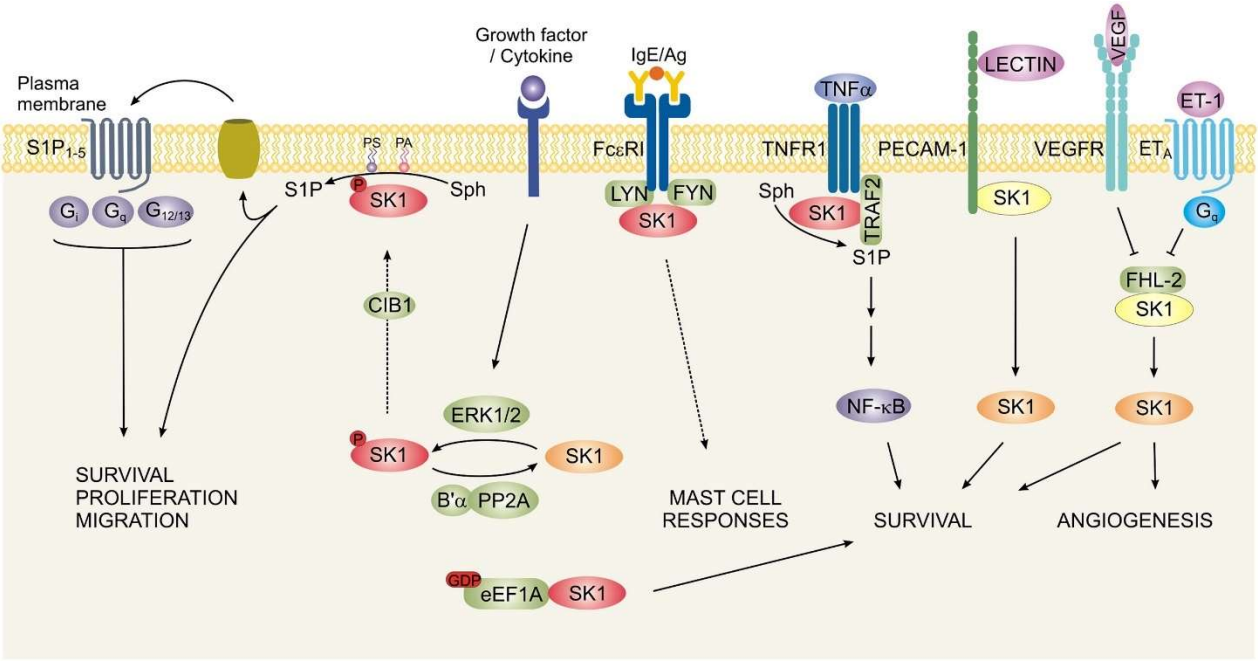

Figure 19 Post-translational regulation of SK1 (Chan and Pitson 2013)

In mast cells, two SRC family protein tyrosine kinases (PTKs), Lyn and Fyn, have been displayed to interact with and directly enhance the catalytic activity of SK1 (Urtz et al. 2004; Ana Olivera et al. 2006). This Lyn/Fyn–SK1 interaction is implicated in IgE-Ag-mediated SK1 activation (Fig. 19). Further studies have ascertained that δ-catenin and eukaryotic elongation factor 1A (eEF1A) can also interact and activate SK1 *in vitro* and in cells (Fujita et al. 2004; Leclercq et al. 2008). The interaction between δ-catenin and SK1 has been linked with increased cell motility, which appeared mediated by augmented SK1 activity since the treatment with an SK inhibitor, *N*,*N*-dimethylsphingosine, abrogated this process (Fujita et al. 2004). Instead, the interaction between SK1 and eEF1A seems to be related to cell survival and proliferation (Leclercq et al. 2008). TRAF2 an adaptor protein associated with TNF-α receptor 1, has also been shown to associate with SK1 and increase cellular SK1 activity when overexpressed (Xia et al. 2002; Alvarez et al. 2010). It is assumed, that this SK1–TRAF2 interaction is crucial for TNF-α-induced activation of the pro-survival, pro-inflammatory transcription factor NF-Kb (Xia et al. 2002; Alvarez et al. 2010) (Fig. 19). In the last decade, several studies have identified several proteins which interact with SK1 and inhibit its catalytic activity. Although the physiological importance of most of these interactions has not been demonstrated, given that SK1 possess intrinsic enzymatic activity, it is reasonable to assume that these 'inhibitors' of SK1 may have a role in avoiding improper production and accumulation of S1P in cells. For example, FHL-2 (Sun et al. 2006; Hayashi et al. 2009) and PECAM-1 (Fukuda et al. 2004) have been demonstrated to inhibit SK1 activity by interacting with this enzyme in cardiomyocytes and vascular

endothelial cells, respectively (Fig. 19). However, after stimulation by extracellular signals, SK1 dissociates from these inhibitory complexes and becomes more active. Other proteins, including SK1 interacting protein (SKIP) (Lacaná et al. 2002) and aminoacylase 1 (Maceyka et al. 2004) have also displayed to interact with and inhibit SK1, although the biological functions of these interplay are unknown. Subcellular localisation plays a fundamental role in the regulation and signalling functions of many signalling proteins. In past decades, growing evidence has proposed that the cellular localisation of sphingolipids and sphingolipid-metabolising enzymes is fundamental for their roles (Wattenberg, Pitson, and Raben 2006). Under normal condition, SK1 remain in the cytoplasm. However, upon stimulation, SK1 is phosphorylated by ERK1/2 at Ser225 and moves to the plasma membrane (Stuart M. Pitson et al. 2003). This relocalisation of SK1 to the plasma membrane is mediated by CIB1 (Jarman et al. 2010). Like other calcium–myristoyl switch proteins, CIB1 translocates to the plasma membrane by a process dependent on both its myristoylation and calcium binding. In this way CIB1 provides a process for active translocation of SK1 to the plasma membrane following enhance in calcium levels that are associated with SK1 activation. The relocation of SK1 to the plasma membrane is known to give rise to different biological effects, including cell proliferation, survival, and cell motility.

Compared to SK1, significantly less is known about the regulation of SK2. The catalytic activity of SK2 is rapidly increased following exposure to a variety of external stimuli (Alemany et al. 2007) (Fig. 20).

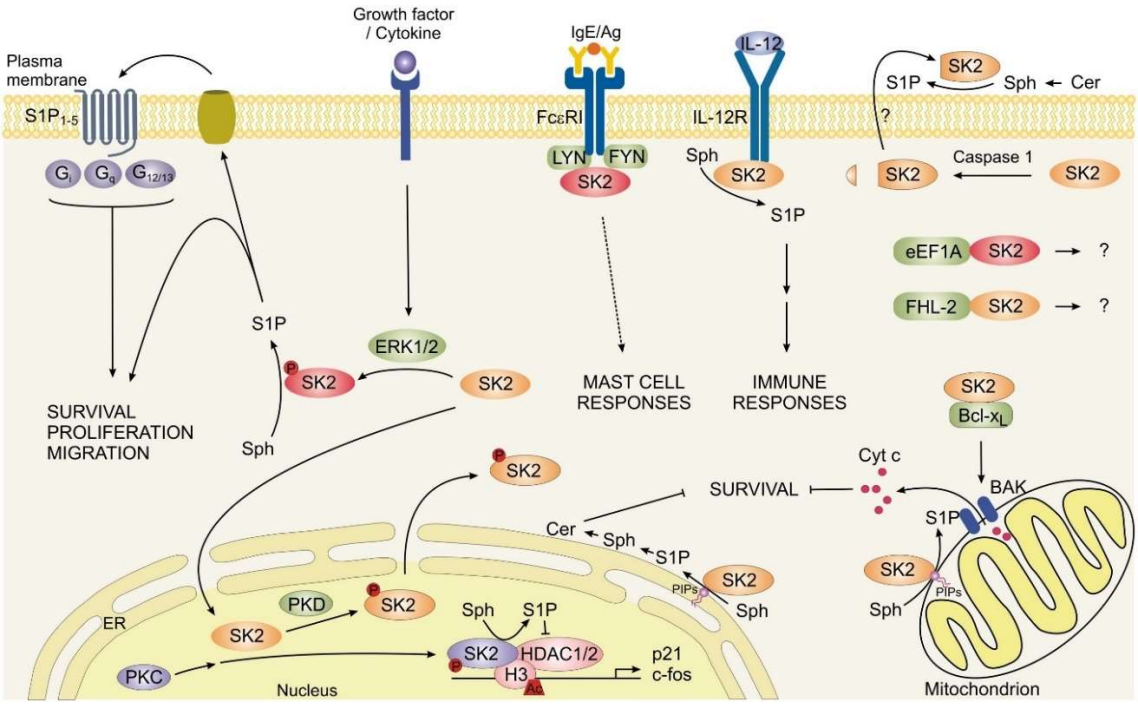

Figure 20 Post-translational regulation of SK2 (Chan and Pitson 2013)

Like SK1, also the activity of SK2 can be regulated by phosphorylation. Interestingly, the region containing the Ser225 phosphorylation site, responsible for SK1 phosphorylation, is not conserved in SK2. Nevertheless, studies have proved that like SK1, SK2 is phosphorylated by ERK1/2 which increases in its catalytic activity (Fig. 20). Two of the sites in SK2 phosphorylated by ERK1/2, are represented by Ser351 and Thr578, which were, therefore, suggested to mediate SK2 activation (Hait et al. 2007). In physiological conditions, SK2 resides principally in the nucleus and cytoplasm. This localisation pattern of SK2 changes under different conditions: enhanced SK2 levels are observed in the ER following serum starvation, and a decrease of SK2 content in the nucleus is a consequence of protein kinase C activation (Maceyka et al. 2005; Ding et al. 2007). While the mechanism responsible for the localisation of SK2 to the ER is unclear, its localisation to the nucleus is thought to be mediated by functional nuclear localisation and export signals (Igarashi et al. 2003). Of note, studies have shown that phosphorylation of SK2 at either Ser383 or Ser385 within its nuclear export signal sequence by protein kinase D seems to mediate its nuclear export (Ding et al. 2007). Besides its overlapping roles with SK1, SK2 also appears to generate specific signalling pools of S1P. For example, SK2-derived S1P has been involved in uterine deciduation (Mizugishi et al. 2007), histone deacetylase-mediated transcriptional regulation (Hait et al. 2009), macrophage polarization and tumour association (Weigert et al. 2009). It is known that SK2 also mediates effects independently of S1P. For example, SK2 contains a BH3-domain that can be responsible for its induction of apoptosis in an S1P receptor-independent mode that implicates its direct interaction with Bcl-xL, release of cytochrome c, and activation of caspase-3 (H. Liu et al. 2003) (Fig. 20). Of note, recent studies have suggested that the apoptotic roles of SK2 are mediated by its mitochondrial localisation, facilitating S1P-induced mitochondrial outer membrane permeability through modulation of the pro-apoptotic Bak protein (Chipuk et al. 2012). A recent work has demonstrated an association of nuclear localised SK2 with the histone H3–HDAC1/2 complex, proposing a role of SK2 in epigenetic regulation (Hait et al. 2009) (Fig. 20). Indeed, overexpression of SK2 enhanced acetylation on specific lysine residues of histones H3, H4 and H2B (Hait et al. 2009). Furthermore, the same study also demonstrated that HDAC1/2 is a direct intracellular target of S1P, with S1P-mediated inhibition of HDAC1/2 activity resulting in increased transcription of cyclin-dependent kinase inhibitor p21 and the transcriptional regulator c-fos (Fig. 20). Interestingly, these SK2-dependent effects, including increased association with HDAC1/2 and enhanced level of nuclear S1P, were major following treatment with phorbol esters, known activators of protein kinase C which enhances the phosphorylation and catalytic activity of SK2 (Hait et al. 2009). Many SK1 interacting proteins have also been shown to interact with SK2, including calmodulin (CaM) (Sutherland et al. 2006), Lyn and Fyn (Ana Olivera et al. 2006) and eEF1A (Leclercq et al. 2008) (Fig. 20).

During the last several years, it has become clear that these enzymes have many important roles like cell growth, survival (A. Olivera et al. 1999) and angiogenesis (Anelli et al. 2010). The SK1/SK2 double knockout mouse is embryonic lethal due to severe defects in development, including angiogenesis and neurogenesis (Mizugishi et al. 2005). Nevertheless, either SK1 or SK2 single knockout mice develop and reproduce normally. This finding suggests that each isoform of SKs may at least partially compensate for the absence of the other and have at least some functional redundancies in mice. In agreement with this, both SK1 and SK2 seem to be important for EGF-induced migration of breast cancer cells (Hait et al. 2005), and TGFβ-induced migration and invasion of oesophageal cancer cells (Miller et al. 2008). Both SKs isoforms have also been found within the centrosome, where it is assumed they may play a role in regulating spindle formation and mitosis (Gillies et al. 2009).

1.6.4 Sphingosine lyase (SPL)

SPL is the terminal enzyme in the sphingolipid degradative pathway and an essential regulator of S1P as well as the levels of other sphingolipid intermediates, which influence many aspects of cell growth, proliferation, and death. SPL is a single pass transmembrane protein showing type I topology, and is exclusively localized to the ER (M. Ikeda, Kihara, and Igarashi 2004). The catalytic site of SPL faces the cytosolic surface of the ER, where it has access to cytosolically produced S1P. The enzyme needs the coenzyme pyridoxal 5'- phosphate; has a pH optimum of 7.4–7.6; and is inhibited by heavy-metal ions (Fig. 21).

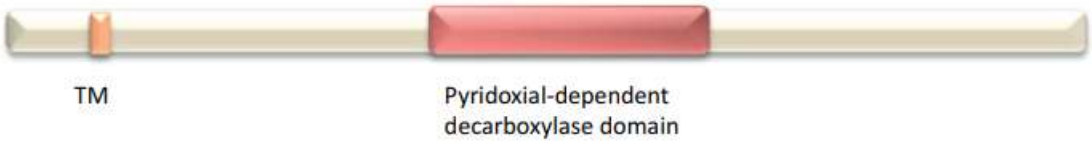

Figure 21 Sphingosine 1-phospate lyase (SPL) (O'Sullivan and Dev 2013)

Activity is specific for the D-erythro isomers of phosphorylated sphingoid bases, including S1P, dihydrosphingosine 1-phosphate and phytosphingosine 1-phosphate, although chain length can differ (Serra and Saba 2010). The enzyme is ubiquitously expressed in species and mammalian tissues, with its highest expression in the thymus and intestines, and its lowest expression in the brain and skeletal muscle. Moreover, no SPL activity can be found in platelets or red blood cells since these cells lack ER membranes (M. Ikeda, Kihara, and Igarashi 2004). The identification of the *sgpl1* gene in yeast (Saba et al. 1997) and after in mammals, indicates that phosphorylated sphingoid base metabolism is

a process that is conserved during the evolution. Deletion studies, have shown the vital role of SPL and phosphorylated sphingoid bases in the regulation of responses to nutrient deprivation in yeast (Gottlieb, Heideman, and Saba 1999). Disruption of the *sgpl1* gene in slime, mould affects several phases of development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating 'slugs,' and terminal spore differentiation, implicating SPL in many processes in multicellular development (G. Li et al. 2001).

1.6.5 S1P phosphatases (SPPs)

SGPPs were first identified in yeast and shown to be fundamental regulators of the heat-stress response (C. Mao, Saba, and Obeid 1999). Homology with yeast genes led to the discovery of genes encoding two mammalian *SGPPs*, *SGPP1* (Le Stunff, Peterson et al. 2002) and *SGPP2* (Ogawa et al. 2003), both containing three conserved motifs (Fig. 22). The latter belongs to the family of magnesium-dependent, N-ethylmaleimide-insensitive type 2 lipid phosphate phosphohydrolases (LPPs) (Stukey and Carman 1997).

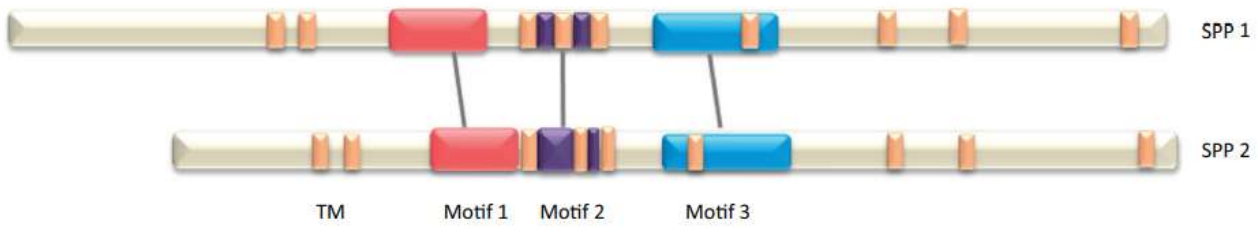

Figure 22 Sphingosine 1-phosphate phosphatases (SPPs) (O'Sullivan and Dev 2013)

Both mammalian SPPs proteins localize to the ER, reduce S1P and dihydroS1P levels by catalyzing their dephosphorylation, and induce apoptosis on overexpression by increasing the formation of Cer (Le Stunff, Galve-Roperh, et al. 2002). SPP1 is highly specific for sphingoid base phosphate esters, like S1P, dihydrosphingosine-1-phosphate (dihydroS1P) and phytosphingosine-1-phosphate (phytoS1P) (Mandala et al. 2000; Le Stunff, Peterson, et al. 2002). Notably, overexpression of SPP1 results in an enhancement of Cer accumulation, suggesting that dephosphorylation of S1P is a rate limiting step in the salvage pathway (Mandala et al. 2000; Le Stunff, Galve-Roperh, et al. 2002). Therefore, the regulation of SPP1 levels can modify the metabolic fate of S1P to be primarily recycled into Cer. In recent years, SPP1 conversion to Cer was shown to be increased by overexpression of SK2, proposing that SK2 and SPP1 work coordinately to enhance Sph salvage (Le Stunff et al. 2007).

In addition, higher expression of SPP1 reduced extracellular release of S1P, indicating that SPP1 can negatively regulate extracellular signalling of S1P (Johnson et al. 2003). SPP2 present a 70% of similarity to SPP1. It was shown that SPP2 is upregulated during inflammatory responses, however, it is unclear what effect this has on sphingolipid metabolism (Mechtcheriakova et al. 2007). It remains to be clarified if SPP2 plays an important role in regulating the sphingolipid recycling pathway like SPP1. While SPP2 is also highly specific for sphingoid base phosphate esters, it has different properties than SPP1, like sensitivity to several phosphohydrolase inhibitors. Both SPP isoforms are expressed ubiquitously with high expression in the kidney. On the other hand, SPP1 is highly expressed in the placenta whereas, SPP2 is highly expressed in the heart (Ogawa et al. 2003).

1.6.6 Intracellular targets of S1P

The intracellular targets of S1P produced by SK1 and SK2 differ, likely due to the distinct subcellular localisation of the two SK isoforms and target effector proteins. As previously described, SK1 is predominantly cytoplasmic and translocates to the plasma membrane to access sphingosine, whereas SK2 shuttles to and from the nucleus (Maceyka et al. 2005).

SK1-derived S1P binds to the RING domain of TRAF2, an E3 ligase which associates with SK1, thus acting as a cofactor in the TRAF2-catalysed Lys63-polyubiquitination of RIP1, a signalling platform in the NF-κB pathway regulating cell survival, inflammatory and immune responses (Alvarez et al. 2010). Furthermore, upon ER stress, S1P–TRAF2–RIP1 complex can associate with the stress-responsive proteins HSP90α, GRP94 and ER to nucleus signalling 1 (IRE1α), and likely also with the E3 ubiquitin ligase STIP1 homology and U-box-containing protein 1 (STUB1) *via* HSP90α, contributing to enhanced RIP1 polyubiquitination and activation of the NF-κB pathway (Park et al. 2016). However, the role of SK1 in TRAF2-NFκB signalling is controversial, as others have obtained conflicting results (Etemadi et al. 2015).

Recently, by *in vitro* studies, it was discovered that in Hela cells intracellular but not GPCR-dependent extracellular S1P activates atypical protein kinase C (aPKC) by directly binding to its kinase domain, resulting in alleviation of autoinhibitory constraints (Kajimoto et al. 2019). aPKC is one of the three PKC subtypes, and it's composed of a regulatory domain (C1), two catalytic domains (C3, C4) for substrate binding and Pseudo and PB1 domains at its amino terminal region. S1P-mediated aPKC activation protects cells from apoptosis (Kajimoto et al. 2019).

Recently, a new role for SK1/S1P in the regulation of proper endosomal processing/endocytic signalling and neurotransmission has been reported (H. Shen et al. 2014). The artificial alteration of the cholesterol/sphingomyelin balance in the plasma membrane, leads to the formation of clusters of

narrow endocytic tubular invaginations, which are positives for N-BAR proteins. SK1 is co-localised in these tubules by the interaction of a hydrophobic patch on the enzyme surface, in a manner sensitive to the curvature of the membrane with the lipid bilayer. In addition, knockdown of SK1 produces endocytic recycling defects and only wild type SK1 but not a hydrophobic patch mutant V268Q-SK1, rescued neurotransmission defects of the loss-of-function mutants (H. Shen et al. 2014). The role of SK1 in regulating endosomal signalling may have an impact on current views concerning 'inside-out' signalling (TAKABE et al. 2008). At mitochondria level, SK2-derived S1P binds to the main inner mitochondrial membrane protein, prohibitin 2 (PHB2), which regulates mitochondrial assembly and function. Depletion of SK2 or PHB2 leads to dysfunctional mitochondrial respiration at the level of cytochrome-c oxidase (Strub et al. 2011). In addition, the hearts of *Sk*2−/− mice are not protected from ischaemic injury by preconditioning, unlike wild type mice, and knockdown of SK2 or PHB2 or cytochrome c oxidase in cardiomyocytes similarly suppressed cytoprotection by preconditioning (Gomez et al. 2011). This evidence, therefore, suggests that the interaction of mitochondrial S1P with homomeric PHB2 is important for cytochrome-c oxidase assembly, mitochondrial respiration and cytoprotection. In contrast, SK2-derived S1P has been described to cooperate with the mitochondrial proapoptotic protein BAK, affecting cytochrome c release upon altered mitochondrial outer membrane potential (Chipuk et al. 2012). Therefore, the role of mitochondrial S1P may be cell context dependent. An additional role of SK2-derived S1P is the stabilisation of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, which maintains telomeres and is often increased in activity in cancer cells. Computer modelling and mutagenesis demonstrated that the C′3-OH of S1P binds with D684 in hTERT and that mutation of this residue or deletion of SK2 reduced hTERT stability, telomere integrity and promoted senescence. The binding of S1P to hTERT prevents its interaction with the E3 ligase makorin ring finger protein (MKRN1), which ubiquitinates hTERT and targets it for proteasomal degradation (Panneer Selvam et al. 2015). Notably, wild type hTERT, but not S1P-binding deficient hTERT restores tumour growth when SK2 was pharmacologically inhibited. S1P binding to hTERT suggested to imitate its phosphorylation, which normally stabilise telomerase to improve tumour growth (Panneer Selvam et al. 2015). Therefore, targeting SK2 with inhibitors may be effective in cancer treatments to eliminate replicative immortality.

HDAC1 and HDAC2 are direct targets of S1P. Nuclear SK2 and SK2-generated S1P directly bind with HDAC1 and HDAC2 in a co-repressor complex at the promoter regions of cyclin-dependent kinase inhibitor p21 or the transcriptional regulator c-Fos, inhibits HDACs, enhance acetylation of histone H3 and subsequently enhance transcription (Hait et al. 2009). Reduced HDAC activity caused by nuclear S1P accumulation in *Sgpl1*-deficient mouse embryonic fibroblasts is also linked with

dysregulation of Ca^{2+} homeostasis (Ihlefeld et al. 2012). Multiple lines of evidence have established an association between S1P-mediated inhibition of HDAC with the development and function of the central nervous system. *Sk2*−/−mice, with reduced levels of S1P and dihydro-S1P as well as histone acetylation in the hippocampus, exhibit a defect in memory function and contextual fear extinction, which can be rescued by the HDAC inhibitor suberoylanilide hydroxamic acid (Hait et al. 2014). These data suggest that the nuclear S1P–HDAC axis may play an important role in nervous system development and function, probably through epigenetic regulation of gene expression.

Moreover, cytoplasmic S1P has been suggested to bind to peroxisome proliferator-activated receptor gamma (PPARγ), enhancing the expression of genes regulated by this transcription factor. S1P-regulation of PPARγ was suggested to be involved in vascular development, which is reduced in *Sk1*−/−/*Sk2*+/−mice, and may be targeted therapeutically to manipulate neovascularisation (Parham et al. 2015). However, it remains to be determined the identity of the SK isoform responsible for the S1P-dependent regulation of PPARγ.

BACE1 is the enzyme involved in production of amyloid-β peptide (Aβ/APP) in the nervous system, which is the main cause of Alzheimer's disease. APP is usually endoproteolysed by α-secretase to sAPP-α, with C83 proteolytic product subsequently cleaved by γ-secretase to produce P3 and APP intracellular domain (AICD). However, under pathological conditions, APP is endoproteolysed by BACE1 (β-secretase) to sAPP-β and C99 proteolytic product, and C99 then is cleaved by γ-secretase to form Aβ and AICD. Takasugi et al. (Takasugi et al. 2011) demonstrated that S1P directly promotes BACE1 activity by binding to full-length BACE1, therefore either down-regulating SK1 or up-regulating SPL in mice decreases BACE1 activity and consequently reduces the production of Aβ (Takasugi et al. 2011, 1). *Sgpl1* deficiency-induced S1P accumulation that, in turn, impairs lysosomal degradation of APP and amyloidogenic C-terminal fragments, and this deficit can be partially restored by selective mobilization of Ca^{2+} from ER or lysosomes (Karaca et al. 2014). Together, S1P signalling components may provide promising therapeutic targets for Alzheimer's disease.

1.6.7 S1P signalling via G protein-coupled receptors

Extracellular S1P binds with high affinity to five specific transmembrane G protein-coupled receptors (GPCRs), designated $S1P_{1-5}$, in an autocrine or paracrine manner, mediating cellular activity through downstream signalling molecules (Alvarez, Milstien, and Spiegel 2007) (Fig. 23). GPCR is bound to the α subunit of a heterotrimeric G protein (Gα), passing from the inactive to the active state guanosine triphosphate (GTP) bound. Gα proteins are classified into four different isoforms: Gs, Gi, Gq and G12 / 13 (Okashah et al. 2019). S1PRs have distinct preferences for protein G, with $S1P_1$ exclusively

coupling to Gi, $S1P_2$ and $S1P_3$ to Gi, Gq and G12 / 13 and $S1P_4$ and $S1P_5$ to Gi and G12 / 13 (Siehler and Manning 2002). Therefore, in response to receptor activation, activation of specific signalling pathways occurs, such as pathways mediated by small GTPases of the Rho family (Rac and Rho) (Jo et al. 2005; Takashima et al. 2008), adenylated cyclase (Jiang et al. 2007), Jun N-terminal kinase (JNK) (Y. M. Kim et al. 2011), phospholipase C and intracellular calcium (Björklund et al. 2005), as well as Akt and ERK1 / 2 (M.-J. Lee et al. 2001) (Fig. 23). Consequently, the net result of S1P signalling *via* S1PRs is the regulation of a wide range of cellular processes, such as cell survival, motility, and angiogenesis in normal tissues (Brinkmann 2007). S1PRs are canonical members of the rhodopsin (Class A) family of GPCRs, with typical structural features, including a small extracellular N-terminal domain (30-50 residues), 7 helical transmembrane domains, and an intracellular C-terminal domain.

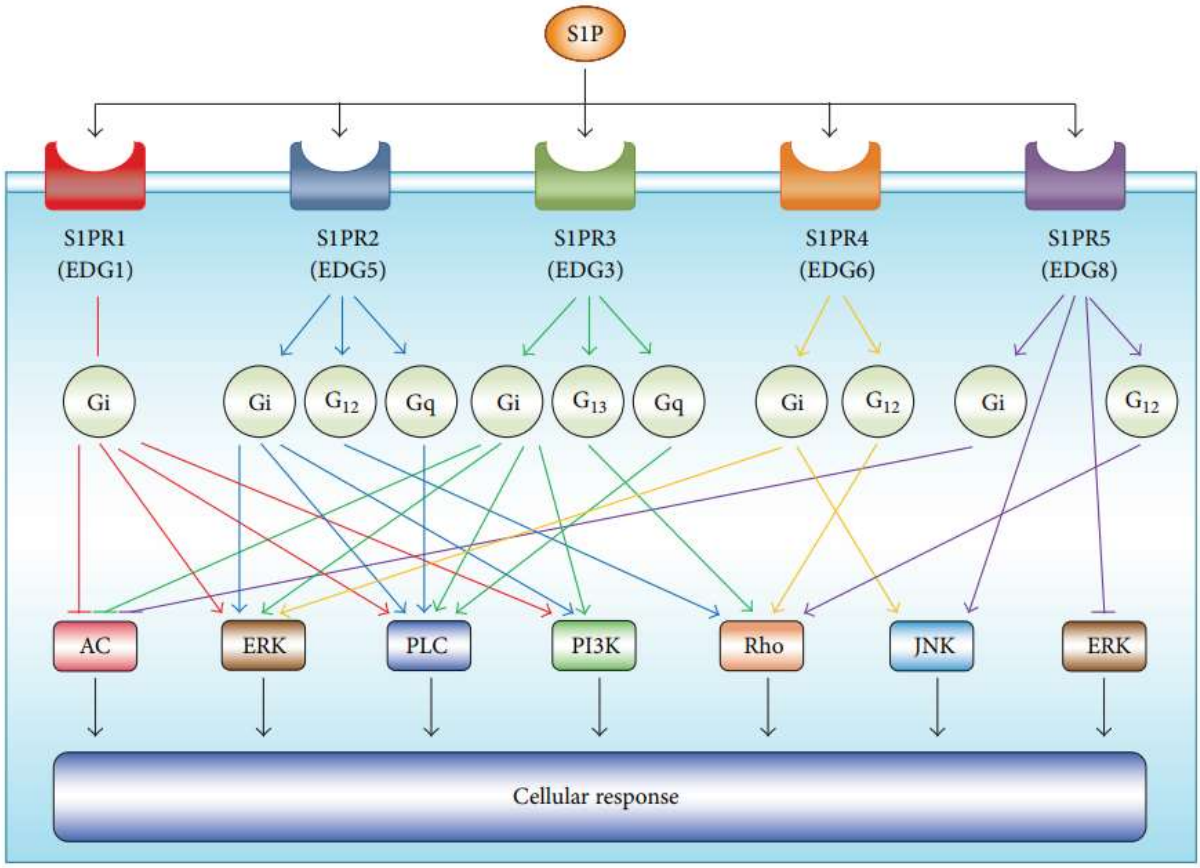

Figure 23 Signalling pathways of S1P receptors (Nagahashi et al. 2014)

$S1P_1$ represents the first member of the S1PR family originally identified as an inducible transcript during *in vitro* differentiation of human umbilical vein endothelial cells (HUVEC) (T Hla and Maciag 1990). Numerous studies have shown that many different mouse and rat tissues, such as the brain, heart, lung, liver, kidney, uterus, and testis, express the mRNA for $S1P_1$ (Lado et al. 1994; C. H. Liu

and Hla 1997). Furthermore, $S1P_1$ expression appears to be up-regulated during the embryonic development of the mice, reaching maximum values by day 15.5, when strong signals could be found in the ossification centers, liver, lung and pulmonary trunk of the mice (C. H. Liu and Hla 1997). $S1P_1$ binds exclusively to the heterotrimeric G protein, Gi, differentiating itself from other S1PRs that can bind to multiple classes of G proteins (Windh et al. 1999). Overall, $S1P_1$, thanks to its widespread expression in different cell types and tissues and various stages of development, possesses several physiological functions. For example, $S1P_1$ is known to play a crucial role in angiogenesis. Indeed its deletion in mouse embryos is found to be lethal due to haemorrhage resulting from incomplete vascular maturation, as smooth muscle cells and pericytes were unable to migrate and envelop the nascent endothelial tubes (Y. Liu et al. 2000). Furthermore, $S1P_1$ play a critical role in the regulation of the cytoskeleton. Its expression was found to be necessary for S1P-induced cortical actin localization at intercellular junctions in HUVEC cells (M.-J. Lee et al. 1999). Consistent with this work, it was recently shown that $S1P_1$ can promote the integrity of the endothelial cell barrier through a mechanism involving S1PR-dependent cytoskeletal changes (Xiong and Hla 2014). The study by Okamoto and colleagues (Okamoto et al. 2000) demonstrated that S1P could increase membrane ruffling in a Rac-dependent manner in $S1P_1$ transfected CHO cells but not $S1P_2$ transfected CHO cells. This peculiarity of $S1P_1$ is caused by an RxRxxT / S consensus sequence in its third intracellular domain, which is not present in the other isoforms of S1PRs. This residue is phosphorylated by protein kinase B / Akt, leading to Rac activation, cortical actin assembly and cell migration (M.-J. Lee et al. 2001). Furthermore, $S1P_1$ has been shown to stimulate pathways involving the rat sarcoma (Ras) family of small GTPases and ERKs, increase proliferation, and activate the phosphatidylinositide 3-kinase (PI3K) / Akt (protein kinase B) to inhibit apoptosis (O'Sullivan and Dev 2013). Finally, $S1P_1$ has been observed to play an important role in the regulation of immune cells. In fact, its expression is necessary for lymphocytes exit from the lymph nodes (Matloubian et al. 2004) and is differentially regulated during the development of the immune response. Upregulation of $S1P_1$ allows T lymphocytes to migrate from the thymus to lymph nodes, where the receptor is downregulated to mediate retention of T lymphocytes in secondary lymphoid organs (Matloubian et al. 2004). Inhibition of this receptor by the immunosuppressive drug FTY720 is beneficial for patients with relapsing multiple sclerosis (Yeh and Weinstock-Guttman 2011). This compound is an Sph analog that binds all S1PRs except $S1P_2$ after being phosphorylated by SK2. FTY720-mediated activation of $S1P_1$ leads to its ubiquitin-dependent degradation, which causes lymphocyte

sequestration and thus immunosuppression (Zhi et al. 2011). It is currently used in the clinic and is known as fingolimod (Yeh and Weinstock-Guttman 2011).

$S1P_2$, was originally cloned from cDNA libraries obtained from rat brains, and later from rat vascular smooth muscle cells, thanks to the study of two different laboratories (Okazaki et al. 1993; Lado et al. 1994). Like $S1P_1$, expression of $S1P_2$ is also present in many tissues and cell types. For example, $S1P_2$ mRNA was detected in the brain, heart, lung, gastrointestinal tract, liver, kidneys, uterus, and testes (Adada et al. 2013). One of the main actions in which $S1P_2$ is involved is the regulation of capillary paracellular permeability. $S1P_2$ coupling to the Rho - Rho protein kinase pathway (ROCK) leads to the activation of the PTEN phosphatase, thus inhibiting the PI3Kpathway (Sanchez et al. 2007). This, in turn, results in the formation of stress fibers and disrupts the adherent junctions between endothelial cells. It is also known that $S1P_2$ can modify the endothelial function concerning wound healing. In particular, increased expression of $S1P_2$ has been reported in senescent endothelial cells (Lu et al. 2012), which would be responsible for their malfunction, due to reduced tube formation and migration of the endothelial cells themselves. New evidence is emerging on $S1P_2$ functions in the liver and pancreas, suggesting critical metabolic roles for this receptor (Imasawa et al. 2010). $S1P_2$ blockade was observed to be involved in protection of streptozotocin-induced apoptosis of pancreatic β-cells and progression of diabetes. Indeed, $S1P_2$ -/- mice, were found to be protected from pancreatic beta cell apoptosis, presenting higher insulin and lower glucose (Imasawa et al. 2010). It has also been described, how conjugated bile acids activate ERK1 / 2 and AKT signalling pathways, mainly through $S1P_2$ in primary rodent hepatocytes (Studer et al. 2012), regulating multiple hepatic metabolic pathways, including glucose control, synthesis of bile acids and lipid metabolism. The actions of $S1P_2$ on hepatocytes are not only limited to the regulation of their metabolic functions, but also extended to the regulation of hepatocyte regeneration after injury (H. Ikeda et al. 2009). Indeed, the liver of $S1P_2$ -/- mice exhibited less fibrosis and greater regeneration after hepatic injection of carbon tetrachloride, to induce the hepatotoxic pattern. Furthermore, $S1P_2$ has been implicated in chemotaxis (Takashima et al. 2008), proliferation (Shimizu et al. 2007), differentiation (Medlin et al. 2010), and contraction (Chiba et al. 2010) of muscle cells. Together with $S1P_3$, $S1P_2$, is responsible for the calcium peak observed in myoblasts after treatment with S1P (Meacci et al. 2002). In contrast, in vascular smooth muscle cells, researchers reported that S1P inhibited their migration through action on $S1P_2$ (Takashima et al. 2008). The mechanism underlying this process is the coupling of the receptor to Gq and G12 / 13, to activate the small GTPase Rho and inhibit Rac, resulting in the arrest of cell migration (Takashima et al. 2008). Depending on the tissue of origin, $S1P_2$ stimulation has been shown to cause smooth muscle contraction (Hoefer et al. 2010; Chiba et al. 2010), with different mechanisms. For example, in bronchial smooth muscle cells, S1P / $S1P_2$-mediated

bronchoconstriction occurs *via* the Rho / ROCK pathway (Chiba et al. 2010), while in mesenteric vascular smooth muscle, S1P / $S1P_2$-mediated vasoconstriction occurs *via* Rho-independent activation of the p38-MAPK pathway (Hoefer et al. 2010). $S1P_2$ in the brain, is mainly expressed in the hippocampus, and its absence has been observed in animal models to result in extensive gliosis in this area. Thus, the mice were not only susceptible to lethal seizures, but also suffered from functional CNS impairments such as spatial working memory deficit and increased anxiety (Akahoshi et al. 2011). An interesting study revealed that the blockade of $S1P_2$ by a specific inhibitor, JTE013, significantly increases the migration of neural progenitor cells to areas of cerebral infarction (Kimura et al. 2008).

$S1P_3$ was initially cloned from a human genomic library during a search for cannabinoid receptors using degenerate PCR primer (Yamaguchi et al. 1996). The amino acid sequence has, indeed, 30% homology for the human cannabinoid receptor type 1. Walter and colleagues initially observed that mice with low $S1P_3$ expression had impaired angiogenic function. Subsequently, scientists demonstrated that S1P or FTY720 restored the angiogenic activity of endothelial progenitor cells in culture, activating $S1P_3$ / SRC kinase and CXCR4-dependent JAK2 signalling (Walter et al. 2007). Recent studies have shown that HDLs induce VEGF receptor 2 expression and activation *via* an S1P / $S1P_3$-dependent mechanism, thus promoting human umbilical vein endothelial cell tube formation (Jin et al. 2018). Furthermore, a year ago, Yasuda and colleagues observed that the axis S1P / $S1P_3$ could increase angiogenesis in the mouse cornea by increasing the expression of VEGF-A (Yasuda et al. 2021). Several studies have suggested that $S1P_3$ activation in endothelial cells can promote nitric oxide (NO) production to induce vasodilation (Nofer et al. 2004), while in vascular smooth muscle cells can determinate vasoconstriction (A. Murakami et al. 2010). In endothelial cells, HDL or S1P promote the intracellular mobilization of Ca^{2+} and the activation of Gi / PI3K / Akt signalling *via* $S1P_3$, resulting in the phosphorylation of eNOS (endothelial nitric oxide synthase) and the production of NO, thus leading to vasodilation (Nofer et al. 2004). In vascular smooth muscle cells, on the other hand, the S1P / $S1P_3$ axis induces the contraction of this cell type, increasing the concentration of intracellular Ca^{2+} and activating Rho (A. Murakami et al. 2010). It has also been shown that $S1P_3$ signalling plays a critical role in protecting the heart from ischemia reperfusion injury (IRI). Indeed, it was observed that administration with HDL / S1P or pre-treatment with a specific agonist of $S1P_3$ decreased the area of IRI induced myocardial infarction. Furthermore, *in vitro*, and *in vivo* studies have shown that the NO-dependent pathway mediated by $S1P_3$ and the activation of Akt cardiomyocytes protected against apoptosis (Theilmeier et al. 2006; Means et al. 2007; Yung et al. 2017). Fibrosis is a complex and multifactorial disease and mounting evidence suggests that $S1P_3$ is a fibrotic mediator. Various studies show that $S1P_3$ participates in cardiac

fibrosis by activating several intracellular signalling pathways, while receptor deletion significantly reduces the area of cardiac fibrosis in SK1-overexpressing mice (Takuwa et al. 2010). The mechanisms by which $S1P_3$ contributes to the progression of fibrosis can be manifold. For example, it has been found that the S1P / $S1P_3$ axis can stimulate the proliferation of ventricular fibroblasts by activating the SUR2 / Kir6.1 channel or promote their migration after their differentiation into myofibroblasts (Benamer et al. 2011). $S1P_3$ can also promote the development of pulmonary fibrosis by regulating the biological functions of lung fibroblasts and EMT (K. Murakami et al. 2014; Gong et al. 2020). In fact, studies have shown that in patients with idiopathic pulmonary fibrosis, TGF-β1-mediated differentiation of human lung fibroblasts and EMT of alveolar epithelial cells occurs by the $S1P_{2,3}$-G12 / 13 / Rho / ROCK signal (Kono et al. 2007; Milara et al. 2012); and that the S1P concentration is high. *In vivo* studies have shown that $S1P_3$ deficiency was able to alleviate radiation-induced lung inflammation and fibrosis, targeting CTGF or miR-495-3p, respectively. In recent years, it has been shown that hepatic stellate cells, bone marrow-derived mesenchymal stem cells (BMSC) and bone marrow-derived mononuclear / macrophage (BMM) are involved in liver fibrosis. In *in vitro* models, the migration effect, fibrosis activation (α-SMA and pro-collagen I / III expression) and angiopoietin-1 (Ang1) expression of human hepatic stellate cells, induced by S1P and mediated by $S1P_1$ / $S1P_3$, were observed. Blocking these receptors significantly reduced pathological angiogenesis and liver fibrosis in bile duct ligated mice (Xihong Liu et al. 2011; L. Yang et al. 2013). Other studies have suggested that $S1P_{2,3}$-Gi / PI3K / Rac1 signalling mediates BMM migration and recruitment *in vitro*; and demonstrated that administering the $S1P_2$ or $S1P_3$ antagonist JTE-013 or CAY-10444 *in vivo* was able to reduce BMM recruitment into the fibrotic liver, thereby relieving inflammation and fibrosis in bile duct ligated mice (L. Yang et al. 2015).

Numerous evidence suggests that another role of $S1P_3$ is represented by the regulation of the biological functions of several immune cells, including dendritic (DCs), macrophages and natural killer (NK) cells. Furthermore, $S1P_3$ was found to be essential for the recruitment of NK cells and neutrophils in the injured kidney after IRI (Maeda et al. 2007; Bajwa et al. 2012). *In vitro* studies showed that $S1P_3$ was highly expressed in astrocytes, one of the most abundant cell types in the brain (Healy and Antel 2016). Its upregulation in response to inflammatory stimulation led to the activation of RhoA, increasing the expression of inflammatory genes, such as *iNOS*, *COX-2*, *IL-1β*, *IL-6* and *TNF-α* (Dusaban et al. 2017). The specific pharmacological inhibitor of $S1P_3$ CAY-10444, has been shown to play a protective role in cerebral infarction, spinal cord injury and acute intracerebral haemorrhage *in vivo* (Gaire, Song, and Choi 2018; Tang et al. 2018). $S1P_3$ also plays a critical role in cell proliferation. In mouse embryonic stem cells, binding of S1P to $S1P_{1/3}$ was shown to stimulate the translocation of β-arrestin from the cytosol to the membrane and to activate c-SRC. This in turn

induced the $S1P_{1/3}$ / Flk-1 signalling pathway and led to the activation of downstream molecules, including ERK and JNK. It is hypothesized that the latter could initiate the expression of the cell cycle regulatory protein and exert proliferative effects on the embryonic stem cells of mice (Ryu et al. 2014). Numerous studies have also reported the proliferative effect induced by $S1P_3$ in other cell types, such as in HUVEC, (Jin et al. 2018), mesangial cells (Schwalm et al. 2015) and cardiac progenitor cells c-kit +. In particular, in the latter it was observed that $S1P_{2/3}$ coupling with Gα12 / 13 and RhoA determines the activation of the transcriptional response dependent on the serum response factor / transcription factor linked to myocardin A. The S1P-regulated signalling pathway in cardiac progenitor cells improved myocardial response after injury (Castaldi et al. 2016). Finally, S1P has been shown to mediate cell migration through $S1P_3$. Li, et al (C. Li et al. 2009) observed that $S1P_3$ is required for the migration of S1P-triggered BMSCs. Simón and colleagues (Simón et al. 2015) hypothesized that Müller's glial cells, one of the main types of glial cells in the retina, which play a fundamental role in maintaining normal retinal function (Guidry 2005), could synthesize S1P and thus, induce glial migration through $S1P_3$. Finally, according to Vézina et al (Vézina et al. 2018), $S1P_3$ is necessary for S1P-mediated functional migration of human brain microvascular endothelial cells.

$S1P_4$ was originally cloned from human dendritic cells differentiated *in vitro* and was located on human chromosome 19p13.3 (Gräler, Bernhardt, and Lipp 1998). $S1P_4$ is mainly expressed in lymphoid tissues, including the thymus, bone marrow, spleen, and peripheral leukocytes (Kluk and Hla 2002), consequently, most of the functions attributed to it are described for that particular system. It has been shown that the S1P / $S1P_4$ axis promotes the survival of B and T lymphocytes (Rosen and Goetzl 2005). Further studies carried out on T lymphocytes have also shown that $S1P_4$ inhibits the proliferation of these cells and the production of immunostimulant cytokines, thus mediating immunosuppressive effects (W. Wang, Graeler, and Goetzl 2005). Several studies have also reported that $S1P_4$ stimulation induces ERK1 / 2, activates phospholipase C, and modulates the opening of intracellular calcium stores (Van Brocklyn et al. 2000; Yamazaki et al. 2000). Finally, it has been shown that S1P / $S1P_4$ signalling activates RhoA, inducing a cytoskeletal rearrangement, which in turn leads to the activation of cofilin *via* ROCK and the kinase of the LIM domain. The latter are both involved in actin nucleation and severing of actin fibers and myosin light chains, promoting the contractility of cytoskeletal fibers (Olesch et al. 2017). Further work is needed both *in vitro* and *in vivo* to elucidate the characteristics and signalling functions of this receptor.

$S1P_5$ is the most recent member of the S1PRs family to have been cloned and characterized. This receptor is mainly expressed in the brain, especially in white matter tracts (Im et al. 2000). Furthermore, the expression of $S1P_5$ was detected in the skin and the spleen, although there are

conflicting reports on the expression in the latter organ (Im et al. 2000; Ishii et al. 2001). Contrary to the other receptors, the binding of S1P to $S1P_5$ induces the phosphatase-dependent inhibition of ERK1 / 2, resulting in an anti-proliferative effect (Gonda et al. 1999; Malek et al. 2001). One study demonstrated that stimulation of rat oligodendrocytes with PDGF increases $S1P_1$ expression with concomitant $S1P_5$ downregulation, inducing an amplified mitogenic response (Jung et al. 2007). A recent report noted that $S1P_5$ is present in NK cells. $S1P_5$ deficient mice show aberrant homing of NK cells and their mobilization in inflamed organs (Walzer et al. 2007). Furthermore, $S1P_5$ is considered essential for maintaining the integrity of the blood-brain barrier and its immunological quiescence. Finally, it determines a reduction in inflammation by decreasing the monocyte trans endothelial migration in the brain parenchyma and inhibiting the activation of NF-KB and the subsequent secretion of cytokines (van Doorn et al. 2012).

1.7 SPHINGOLIPID ROLE IN UTERINE DISORDERS

Endometriosis is a disease that currently lacks both a defined etiological mechanism and a clear understanding of the cellular processes that occur as the disease progresses. For this reason, over time, several research groups have based their work on identifying possible signalling pathways involved in endometriosis pathogenesis. Santulli P. et al (Santulli et al. 2012), have demonstrated that S1P metabolism was transcriptionally altered in eutopic (16 endometrial biopsy) and ectopic endometrium (12 OMA, 4 DIE, 2 bladder, 2 vaginal) of women with endometriosis. More specifically, they observed that in the endometrium of healthy women, the mRNA expressions of SPP2 and SPL were the most abundant of the S1P metabolic cascade, consistent with the maintenance of this bioactive lipid at a low physiological level. They also found that SPP2 was expressed 1,000 times more than SPP1, suggesting a preference for this phosphatase within the S1P-endometrium axis. In endometriotic lesions, on the other hand, they showed that SPP2 and SPL were under-expressed 3 to 16 times compared to healthy endometrium, while the mRNA levels of the enzymes involved in its synthesis were unchanged. Finally, changes in S1PR mRNA and protein levels were also documented: $S1P_1$ and $S1P_2$ were upregulated in both ectopic and eutopic endometrium, while $S1P_3$ was downregulated only in ectopic endometrium. The results collected by Santulli et al, support a model in which the SK / S1P / $S1P_1$ axis is enhanced in ectopic endometriotic lesions due to the decrease of S1P catabolism (Santulli et al. 2012). Lee et al (Y. H. Lee et al. 2014), demonstrated the presence of an altered sphingolipid metabolism flux in serum, peritoneal fluid, and endometrial tissue in women affected by endometriosis. In particular, they identified the *in vivo* increase of glucosylceramide in endometriotic women as a result of dysregulated sphingolipid metabolic processing by glucosylceramide synthase in the endometrium. The role of sphingolipids in endometriosis is also sustained by altered levels of sphingomyelin in the peritoneal fluid of OMA patients (Vouk et al. 2016). The involvement of lipid metabolism in endometriosis has been confirmed by several studies. For example, Dutta M. et al (Dutta et al. 2016), used lipidomic mass spectrometry to study alterations in serum lipid profiles in eutopic and ectopic lesions in mouse models. This study identified a reduction in phosphatidylethanolamine and an increase in the concentration of phosphatidylcholine, phosphatidylinositol, and sphingomyelin in the serum of mouse models with endometriosis compared to healthy mouse models. Lee et al (Y. H. Lee et al. 2018) applied mass spectrometry–based sphingolipidomics to characterize the peritoneal fluid of women affected by endometriosis-associated infertility (EAI). Moreover, they assessed functional studies in mice to understand the potential functional roles of sphingolipids in affecting oocyte maturation. Interestingly, they discovered a panel of ceramides that are correlated with EAI and demonstrated

how ceramides affect oocyte maturation, through mitochondrial superoxide production. Subsequently, the *in vitro* work conducted by Yoshino O. et al (Yoshino et al. 2019), provided further confirmation on the involvement of S1P metabolism in endometriosis. In fact, the results of the research showed an important increase of SK1 mRNA in cultures of endometriotic stromal cells (ESC) following stimulation with IL-1 and TGF-β, two factors involved in the onset of endometriosis. Furthermore, it has been observed that the treatment with S1P has an important proliferative action on ESC cells and that this effect was reversed by the use of the $S1P_2$ receptor antagonist JTE013 and the $S1P_1$ / $S1P_3$ antagonist VPC23019. Finally, S1P has been shown to induce the expression of the pro-inflammatory cytokine IL-6 in the same cellular model, suggesting a role of the bioactive sphingolipid in endometriosis-associated inflammation. A recent study (Turathum et al. 2022) investigated the metabolic profiles between cumulus cells (CCs) and mural granulosa cells (MGCs) derived from women affected by endometriosis to determine their correlations with oocyte quality. The results indicate that the metabolites related to palmitic acid, sphingolipid metabolism, and autophagic cell death were increased only in CCs, suggesting that sphingolipid metabolism plays an important role in follicle and oocyte growth.

Uterine fibroids are the most common benign tumors of the uterus in up to 70% of reproductive-age women (Bulun 2013). The pathogenesis of leiomyoma remains unknown. The roles of genetic mutations (Mäkinen et al. 2011; Markowski et al. 2011), hormonal disorders (estrogen-progesterone imbalance), and growth factors have been described (Elizabeth A. Stewart et al. 2016). The involvement of sphingolipid pathways in the origin and progression of uterine fibroids has only recently been investigated. Raymond et al (Raymond et al. 2006) showed that in ELT3 rat uterine leiomyoma cells S1P exerts a proliferative and antiapoptotic role and that SK1 was able to mediate the antiapoptotic effect of endothelin-1. Moreover, in the same cells, it was demonstrated that S1P increased the expression of cyclooxygenase *via* ABCC1 release of S1P and $S1P_2$ engagement (Tanfin, Serrano-Sanchez, and Leiber 2011). An interesting study conducted by Heinonen et al (Heinonen et al. 2017) utilized a global metabolomics approach to discover metabolites and metabolic pathways that are dysregulated in different subtypes of uterine fibroids. Indeed, leiomyomas can be classified on the basis of their genetic triggers: mediator complex subunit 12 (MED12) mutations, high mobility group AT-hook 2 (HMGA2) upregulation, or inactivation of fumarate hydratase (FH). Heinonen et al found a significant decrease in the level of sphingolipids and phosphatidylserines in MED12 mutated fibroids, revealing the complex metabolomic heterogeneity of leiomyomas. Finally, a recent study evaluated the potential of the lipid profiling of blood plasma for the low-invasive diagnosis of fibroids recurrence. The results showed significantly different levels of phospholipids,

sphingomyelins, cholesterol esters and triglycerides between women with uterine fibroids, recurrent uterine fibroids and the control group (Tonoyan et al. 2021).

2. RESULTS

2.1 PAPER 1

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Sphingosine 1-phosphate receptors are dysregulated in endometriosis: possible implication in transforming growth factor β–induced fibrosis.

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In this paper, we have studied the role of S1P signalling in fibrosis associated with endometriosis. We collected tissue sample from women affected by endometriosis undergoing laparoscopic surgery and they were categorized as: OMA (n=15) and DIE (n=30). Control endometrial specimens were collected during diagnostic hysteroscopy from 30 nonpregnant women not affected by endometriosis or other uterine disorders. Primarily we first assessed the involvement of S1P signalling in endometriosis, demonstrating an important increase in SK1 mRNA expression in both DIE and OMA compared to the control endometrium of healthy women. In addition, the mRNA expression of CIB1, a ubiquitous protein involved in the modulation of subcellular localization of SK1 and its membrane translocation, was also higher in endometriotic lesions than in healthy controls. Regarding the expression of the enzyme involved in the irreversible degradation of S1P, SPL, was found to be strongly reduced in DIE and increased in OMA compared to controls, suggesting also an alteration of the catabolism of the sphingolipid. Furthermore, the mRNA expression of Spns2, the specific transporter involved in the extracellular release of S1P, was increased in DIE in respect to healthy endometrium. The study of the expression of S1PRs demonstrated profound differences between the sick and healthy samples. $S1P_1$ was transcriptionally enhanced in OMA compared control endometrium. Interestingly $S1P_1$ expression was decreased in DIE than in OMA samples. Instead $S1P_3$ and $S1P_5$ mRNA levels were significantly increased in both OMA and DIE Considering the key role of TGFβ1 in fibrogenesis and the cross-talk between TGFβ1 and S1P, we also studied mRNA expressions of TGFβ1, α-SMA, and COL1A1 in endometriotic lesions. The results showed an important increased of all three fibrotic markers analysed both in OMA and DIE compared healthy endometrium. Finally, in this work, we have also demonstrated that S1P mediates the ability of TGFβ1 to induce fibrosis and EMT markers in an *in vitro* EMT model of uterine adenocarcinoma

cells. In particular, using RNA interference techniques we showed that SKs and $S1P_{2/3}$ are required for the pro-fibrotic action of TGFβ1 in this role.

Altogether our results demonstrated for the first time that an altered S1P signalling pathway can be implicated in the fibrotic phenotype of endometriotic lesions identifying a new potential target counteracting its fibrotic phenotype.

Paper in appendix

2.2 PAPER 2

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Sphingosine 1-phosphate signaling in uterine fibroids: implication in activin A pro-fibrotic effect.

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On the basis of the crucial role of S1P in fibrotic diseases (Donati et al. 2021), including endometriosis (Bernacchioni et al. 2021), in this paper we have studied the role of S1P signalling pathway in uterine fibroids and its possible cross-talk with activin A. We collected tissue samples from 26 premenopausal women. For each patient, two tissue specimens were obtained during surgery: leiomyoma and normal myometrium. For the first time, we demonstrated an important dysregulation of S1P metabolism and signalling in uterine fibroids: enhanced mRNA and protein expression of SK1 and SK2, the enzymes responsible for S1P biosynthesis, and of S1P receptors $S1P_{2,3,5}$ were showed in uterine fibroids compared with adjacent myometrium.

Moreover, we demonstrated that in immortalized human leiomyoma cells but not in myometrial control cells S1P mediates the profibrotic action of activin A. Indeed, the profibrotic action of activin A was significantly reduced when SK1/2 were inhibited, or $S1P_{2/3}$ blocked in leiomyoma cells. In addition, it was showed that activin A increases the mRNA expression levels of SK1, SK2, and $S1P_2$. These results were also confirmed in both primary leiomyoma and myometrial cells. Finally, it was demonstrated that the treatment of leiomyoma cells with S1P increased the expression of fibrotic markers (fibronectin, COL1A1), at the same extent of activin A.

Altogether, these results demonstrate for the first time a functional cross-talk between activin A and S1P signalling pathways, identifying in the SK/S1P axis a key molecular mechanism in the transduction of the fibrotic action of the cytokine in uterine fibroids, opening new perspectives for original pharmacological targets to fight this disease.

Paper in appendix.

2.3 PAPER 3

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Sphingosine 1-phosphate pathway is dysregulated in adenomyosis.

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Considering the profound dysregulation of metabolism and S1P signalling pathway in endometriosis (Bernacchioni et al. 2020), in this paper we investigated the role of the bioactive sphingolipid in adenomyosis. We collected ectopic endometrium from 27 patients suffering from adenomyosis, and 29 eutopic endometrium from women not affected by uterine disorders, which are used as control specimens. We observed that in pathological samples, the mRNA expressions of SK1 and SPP2 were significantly lower compared to healthy endometrium, while mRNA levels of CIB1 were higher in adenomyosis than in control samples. Finally, the mRNA expression of $S1P_2$ resulted significantly lower in patients than in healthy endometrium, whereas $S1P_3$ expression was significantly higher. In addition, the expression levels of the fibrotic marker αSMA were found to be highly upregulated in adenomyosis respect to control. Interestingly, αSMA expression was shown to be statistically related to $S1P_3$ mRNA levels, suggesting a key role of $S1P_3$ in the onset of fibrosis associated to adenomyosis.

In conclusion, for the first time it was discovered a profound dysregulation of mRNA expression of the genes involved in the metabolism and signalling of the bioactive sphingolipid S1P in women with adenomyosis, associated with an increased expression of the fibrotic marker αSMA.

Paper in appendix.

3. DISCUSSION

S1P is a pleiotropic bioactive sphingolipid which regulates numerous cellular processes fundamental for cell growth, survival, invasion, lymphocyte trafficking, vascular integrity, and production of cytokine and chemokine. Furthermore, S1P signalling dysregulation is implicated in different diseases including inflammation, fibrosis, and cancer.

Endometriosis is a sex hormone–dependent inflammatory disease affecting 10% of the female population of reproductive age. It is a heterogeneous disease with three well-recognized phenotypes: ovarian endometriomas (OMA), superficial peritoneal lesions (SUP), and deep infiltrating endometriosis (DIE). The underlying biological key mechanism of endometriosis include hormonal imbalance, changes on genetic and epigenetic levels, neuroangiogenesis, tissue injury and repair mechanisms, epithelial-mesenchymal transition, and fibroblast to myofibroblast transition. Consequently, inflammation, tissue adhesions and fibrosis arise.

In the first published paper we demonstrated for the first time that S1P metabolism and signalling is dysregulated in endometriosis. Indeed, we found that both OMA and DIE lesions have an up-regulation of SK1 levels, while the marked decreased SPL and increased Spns2 expression only in DIE indicates some differences in the biology of these lesions. The result of this deregulation leads to the enhance of S1P signalling. Interestingly, the increase of SK1 showed in endometriotic lesions is mirrored by more elevated mRNA levels of CIB1, a ubiquitous protein that modulate the subcellular localization of SK1 and its membrane translocation, thus controlling S1P cellular generation (Jarman et al. 2010). Moreover, a marked increase of α-SMA, TGFβ1, and COL1A1 mRNA levels was also reported in both types of endometriotic lesions. A recent study have demonstrated that the balance between SK1 and SPL expression is essential for the development of fibrosis: whereas lack of SK1 protects mice against pulmonary fibrosis, SPL deficiency exacerbates the fibrogenetic processes (Suryadevara et al. 2018). Several evidences suggest that SK1 is involved in the action mechanism of TGFβ in fibroblasts that is crucial for ECM deposition and fibroblast transdifferentiation into myofibroblasts (Yamanaka et al. 2004; Kono et al. 2007; Gellings Lowe et al. 2009). Furthermore, it was demonstrated that transgenic mice overexpressing SK1 developed spontaneous myocardial degeneration and cardiac fibrosis, implying a direct role of SK1 in cardiac fibrosis (Takuwa et al. 2010). Finally, recent works have revealed that SK1 levels are also increased in fibrotic livers (M. Sato et al. 2016) and deficiency of SK1 has been reported to ameliorate markers of hepatic injury (Lan et al. 2018). In the first published paper, we also demonstrated that S1PRs are highly expressed in endometriotic lesions. In particular, $S1P_3$, followed by $S1P_5$, was found to be the most expressed receptor in DIE and OMA lesions compared to the control endometrium. $S1P_3$ has been observed to be involved in the onset of fibrosis in various tissues, such as skeletal muscle (Cencetti et al. 2010), lung (L. S. Huang and Natarajan 2015), kidney (Xiwen Zhang, Ritter, and Li

2018) and heart (Takuwa et al. 2010), while $S1P_3$ deficiency relieves radiation-induced pulmonary fibrosis (Gong et al. 2020). Consequently, it is reasonable to assume a possible implication of S1P in fibrosis associated with endometriosis through $S1P_3$.While $S1P_1$, $S1P_2$ and $S1P_3$ are ubiquitously expressed, the expression of $S1P_5$ is limited to distinct cell types, such as the nervous system and lymphatic cells, respectively (Blaho and Hla 2014). Therefore, the observed increase in $S1P_5$ levels in endometriosis is compatible with the neurogenesis process in OMA and DIE associated with pelvic pain in these patients (Tosti et al. 2015; Gori et al. 2016). Our study also revealed that the mRNA expression of $S1P_1$ was increased in OMA samples. This finding might suggest that $S1P_1$ could mediate the enhanced cell survival, proliferation, and migration, seen in this type of endometriosis, as previously reported in other tissues (Nincheri et al. 2010; Calise et al. 2012). Furthermore, Doyle et al (Doyle et al. 2011) showed that $S1P_1$ is implicated in nociceptive processing. Therefore, $S1P_1$ may play a role in activation of both central and peripheral pain pathways in endometriotic lesions. Finally, the first published paper demonstrated a crucial role of S1P signalling pathway in mediating the pro-fibrotic action of TGFβ1 in a uterine adenocarcinoma model. It is well known the role of S1P in tissue fibrogenesis (Donati et al. 2021). Cencetti et al, previously demonstrated that in skeletal muscle myoblasts, after TGFβ1 stimulation $S1P_3$ becomes the principal expressed receptor and SK1 is enhanced and that the SK1/$S1P_3$ signalling pathway mediates the transdifferentiation of myoblasts into myofibroblasts induced by TGFβ1 (Cencetti et al. 2010). Accordingly, in our work we found a higher mRNA expression of $S1P_3$ in endometriotic lesions, and *in vitro* data demonstrated that TGFβ is able to remodel S1PR signalling, enhancing mRNA levels of $S1P_3$. Moreover, it was demonstrated that TGFβ profibrotic effect was abolished when SKs or $S1P_{2/3}$ were blocked, suggesting that the bioactive sphingolipid mediates the profibrotic effect of TGFβ1. The role of bioactive sphingolipids in endometriosis is supported by several studies. Chrobak et al (Chrobak et al. 2009) demonstrated that endometrial cells from women affected by endometriosis are resistant to ceramide-induced apoptosis and have increased survival after exposure to sphingosine analogues. Santulli P. et al (Santulli et al. 2012), showed that S1P metabolism was transcriptionally altered in eutopic and ectopic endometrium of women with endometriosis, in favour of a decreased S1P catabolism. These data are in contrast with the results obtained in our first paper. The reason for this discrepancy could be related to the small number of cases analyzed by Santulli et al and to their analysis carried out in a pool of OMA and DIE samples. Moreover, the role of sphingolipids in endometriosis is also sustained by the presence of their altered metabolism flux in serum, peritoneal fluid, and endometrial tissue in women affected by endometriosis (Y. H. Lee et al. 2014; Vouk et al. 2016; Y. H. Lee et al. 2018). In addition, *in vitro* experimental studies have confirmed the involvement of S1P in endometriosis, using

endometriotic stromal cells as an experimental model stimulated with IL-1 and TGF-β to induce an endometriotic phenotype (Yoshino et al. 2019).

The main S1PR implicated in fibrosis are $S1P_2$ and $S1P_3$, as they determine the activation of the parallel Rho/ Rho kinase and Smad signalling pathways, which in turn mediate the main pro-fibrotic actions of S1P (Maceyka et al. 2012; Donati et al. 2021). In particular $S1P_3$ is involved in the onset of fibrosis in several tissues, such as skeletal muscle, lung, kidney, and heart (Donati et al. 2021), while $S1P_3$ deficiency attenuates radiation-induced pulmonary fibrosis (Gong et al. 2020).

In summary, results presented in the first paper demonstrate that the metabolism and the signalling of the bioactive lipid S1P are profoundly altered in endometriosis, suggesting that the S1P pathway may be a useful biomarker or innovative pharmacologic target for this disease.

Uterine fibroids represent the most common benign gynecologic tumors diagnosed in up to 70% of white women and more than 80% of women of African ancestry during their lifetime (Giuliani, As-Sanie, and Marsh 2020). Uterine fibroids are characterized by enhanced levels of extracellular matrix (ECM), collagen, fibronectin, and proteoglycans and increased expression of inflammatory cytokines (Chegini 2010; Islam et al. 2013), so it is also defined as a fibrotic disease (Malik et al. 2010). The exacerbated production of ECM determinates rigidity of the structure, causing symptoms such as abnormal bleeding and pain (Islam et al. 2018).

In the second published paper we demonstrated for the first time a significant dysregulation of S1P metabolism and signalling in uterine fibroids. Indeed, we showed an increased mRNA and protein expression of both SKs in leiomyoma respect to adjacent myometrium. It is well known that SK1 and SK2 both plays a critical role in the induction of fibrosis in several tissues (Donati et al. 2021). It was demonstrated that in murine models of kidney fibrosis, SK2 is overexpressed and SK2-deficient mice show less severe fibrosis (Schwalm et al. 2017), and gene deletion of SK2 in bone marrow protects mice from folic acid–induced renal fibrosis (Bajwa et al. 2017). Schwalm et al showed that in SK2 overexpressing mice unilateral ureteral obstruction resulted in exacerbated signs of fibrosis that couple with decreased anti-fibrotic protein Smad7 expression (Schwalm et al. 2017). Furthermore, it has been recently demonstrated that in renal NRK-49F cells TGFβ enhances the expression of SK2 that, collaborating with Fyn, is implicated in kidney fibroblast activation and fibrosis induced by the cytokine via STAT3 and Akt (Zhu et al. 2018). The second paper also demonstrated an up-regulation of $S1P_2$, $S1P_3$, and $S1P_5$ mRNA levels in uterine fibroids compared with adjacent myometrium. It was demonstrated that the S1PR primarily involved in fibrosis are $S1P_2$ and $S1P_3$, as they determine the activation of the parallel Rho/ Rho kinase and Smad signalling pathways, which in turn mediate the main pro-fibrotic actions of S1P (Y. Takuwa et al. 2013). Of note, a novel effect of $S1P_5$ on the inflammatory processes during low-dose bleomycin–induced fibrogenesis has been demonstrated in

murine skin (Schmidt et al. 2017). Accordingly, the antagonism of $S1P_2$, $S1P_3$, and $S1P_5$ could represent new therapeutic target for uterine fibroids.

In the second paper we also demonstrated that in human immortalized leiomyoma cells, S1P was able to induce the expression of fibrotic markers fibronectin and COL1A1 but not in myometrial control cells. Finally, an original finding of this work was that S1P signalling mediates the profibrotic role of activin A. Indeed, we demonstrated that in immortalized human leiomyoma cells but not in myometrial control cells, activin A enhanced the mRNA expression of SK1, SK2, and $S1P_2$. Interestingly, the profibrotic action of activin A was abolished when SK1/2 were inhibited, and $S1P_{2/3}$ were blocked, highlighting therefore, for the first time, a functional cross-talk between activin A and S1P signalling. Activin A is a member of the TGF-β superfamily, which plays important roles in cell proliferation, apoptosis, and metabolism, as well as in mediating inflammation, wound repair, and fibrosis. Many studies have described the presence of activin A in uterine tissue of rats and myometrial cell lines (Ciarmela, Wiater, and Vale 2008), human myometrial and leiomyoma tissue explants (Ciarmela, Bloise, et al. 2011) as well as in human primary myometrial and leiomyoma cells (Islam, Catherino, et al. 2014). In particular, it was demonstrated an increased expression levels of activin A in leiomyoma compared with adjacent myometrial tissue (Ciarmela, Bloise, et al. 2011). The ability of activin A to enhance ECM protein expressions in uterine fibroid cells demonstrates its profibrotic role in leiomyoma growth. Of note, while TNFα increases the expression of activin A in myometrial and uterine fibroid cells (Protic et al. 2016), treatment with the an antinflammatory drug named Tranilast decrease activin A as well as ECM expression in both cell types (Islam, Protic, et al. 2014). While extensive interplay has been previously demonstrated between S1P and TGF-β, in the second paper, for the first time, a functional interplay between activin A and S1P has been shown, discovering new molecular mechanisms by which the growth factor determinates its fibrotic actions in leiomyoma. The role of sphingolipid pathways in the development of uterine fibroids has only recently been demonstrated. Raymond et al (Raymond et al. 2006) showed that endothelin-1 possesses a potent antiapoptotic effect in ELT3 rat uterine leiomyoma cells, that involves sphingolipid metabolism through the activation of SK1. Moreover, in the same cells, it was demonstrated that S1P enhanced the cyclooxygenase expression via ABCC1 release and $S1P_2$ signalling (Tanfin, Serrano-Sanchez, and Leiber 2011). Finally, Heinonen et al (Heinonen et al. 2017) thanks to a global metabolomics approach have discovered an important dysregulation of sphingolipid metabolism in all subtypes uterine fibroids except triple wild-type leiomyomas.

In summary, the second paper show for the first time, a crucial involvement of S1P pathway in the molecular mechanisms driving the fibrotic phenotype in uterine fibrosis. Finally, the discover of

functional cross-talk between activin A and S1P signalling open new perspectives for uterine fibroid treatment.

Adenomyosis is a common chronic gynecological disorder accompanied by progressive dysmenorrhea and infertility. It is characterized by the presence of ectopic endometrial glands and stroma surrounded by hyperplastic smooth muscle within the myometrium (Vannuccini et al. 2017). Adenomyosis and endometriosis share several features and it was observed that, at least in some subgroups, the two conditions often coexist (Lazzeri et al. 2014). The pathogenic mechanisms of adenomyosis development are still unclear; however, it was demonstrated that similar to endometriosis, adenomyotic lesions undergo EMT, FMT, SMM, and progress ultimately to fibrosis (Xishi Liu et al. 2016a; M. Shen et al. 2016; Q. Zhang, Duan, Liu, et al. 2016). Among all the molecular mechanism implicated in adenomyosis progression, EMT appears to be the most documented. Numerous molecules and growth factors have been demonstrated to be involved in EMT in the context of adenomyosis: sex steroid hormones (Y.-J. Chen et al. 2010), β-catenin (Oh et al. 2013), Notch1 (Qi et al. 2015), hepatocyte growth factor (HGF) (Khan et al. 2015), TGF-β1 (M. Shen et al. 2016; Xishi Liu et al. 2016b), Integrin-linked kinase (ILK) (W. Zhou et al. 2018), eukaryotic translation initiation factor 3 subunit e (eIF3e) (Cai et al. 2019), focal adhesion kinase (FAK) (Zheng et al. 2018), and Talin1 (Y.-Y. Wang et al. 2021). It is well known that adenomyotic lesions are also characterized by increased nerve fiber density and hyperinnervation (Xinmei Zhang et al. 2010), and therefore by enhanced secretion of neuropeptides. Interestingly, many of them, such as substance P and calcitonin gene-related peptide (CGRP), can promote EMT, FMT, and SMM in ectopic endometrium (Xishi Liu, Yan, and Guo 2019; Yan, Liu, and Guo 2019), facilitating fibrogenesis.

In consideration of the fibrotic nature of adenomyosis and the key role of S1P in fibrotic diseases, including endometriosis and uterine fibroids, in the third paper we investigated if the bioactive lipid signalling axis was altered in adenomyosis. The results demonstrated a decreased expression of SK1 with concomitant no variations of SK2 levels, suggesting a reduction in pathological samples of the SK1- synthetized S1P pool that can be exported out of the cells and bind to its specific receptors. However, the enhanced levels of CIB1 observed in adenomyosis, may possibly compensate for the decreased expression of SK1, making the enzyme more active. Furthermore, the reduced levels of the specific phosphatase SPP2 suggest its probable role in increasing S1P levels in adenomyotic lesions. This work also demonstrated a profound dysregulation of S1P signalling. The downregulation of $S1P_2$ levels in adenomyosis, suggest a possible role of S1P in the increased migration of adenomyotic cells. Furthermore, the up regulation of $S1P_3$ in pathological samples may play a central role in the development of adenomyosis related fibrosis. Indeed, an interest finding of this paper is the discovery

that increased expression levels of the fibrotic marker αSMA, in adenomyosis, statistically correlated with $S1P_3$ mRNA levels, suggesting that the pharmacological blockade of $S1P_3$ by fingolimod (FTY720), the first orally available agent FDA approved for the treatment of relapsing-remitting multiple sclerosis and efficacious in counteracting fibrosis in many tissues, may be studied in adenomyosis.

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5. APPENDIX

Sphingosine 1-phosphate receptors are dysregulated in endometriosis: possible implication in transforming growth factor β–induced fibrosis

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Objective: To study the molecular mechanisms involved in the appearance of the fibrotic trait in endometriosis by investigating whether the signaling pathway of the bioactive sphingolipid sphingosine 1-phosphate (S1P) was altered in endometriotic lesions.
Design: Case-control laboratory study.
Setting: University research institute and university hospital.
Patient(s): A total of 75 women, with and without endometriosis, were included in the study.
Interventions(s): Endometrial samples were obtained from women affected (n = 15 endometrioma [OMA]; n = 30 deep infiltrating endometriosis [DIE]) and not (n = 30) by endometriosis by means of laparoscopic surgery, followed by clinical and imaging investigation and checking for the expression of fibrosis markers and genes implicated in S1P metabolism and signaling by means of real-time polymerase chain reaction.
Main Outcome Measure(s): The role of the S1P signaling axis in endometriosis-associated fibrosis was studied in vitro, where RNA interference approaches were used to investigate if S1P synthesis by sphingosine kinases (SKs) and specific S1P receptors (S1PRs) are implicated in the profibrotic effect of the cytokine transforming growth factor (TGF) β1.
Result(s): mRNA expression analysis of S1PR demonstrated a deep dysregulation of S1P signaling in endometriosis, characterized by increased expression of fibrosis markers: $S1P_1$ was transcriptionally more expressed in OMA, and $S1P_3$ and $S1P_5$ mRNA levels were significantly augmented in both OMA and DIE. SK1 and its activating protein calcium- and integrin-binding protein 1 (CIB1) were significantly up-regulated in OMA and DIE. A crucial role for the SK/S1PR axis in the profibrotic effect elicited by TGFβ1 was highlighted in vitro.
Conclusion(s): The S1P signaling axis may represent a useful biomarker or innovative pharmacologic target for endometriosis. (Fertil Steril® 2021;115:501-11. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Sphingosine 1-phosphate receptors, peritoneal endometriosis, fibrosis, sphingosine kinase, inflammation

Endometriosis is a sex hormone–dependent inflammatory disease affecting ~6%–10% of women of reproductive age (1). The presence of fibrotic tissue inside and around the endometriotic ovarian and peritoneal lesions leads to tissue adhesions, scarring, and anatomic distortions that may represent one of the causes of pelvic pain. The molecular mechanisms responsible for the development of fibrosis in endometriosis are, however, still elusive. Ovarian endometriosis (endometrioma [OMA]) and deep infiltrating endometriosis (DIE) share most of the same pathogenetic mechanisms, but specific features explain the different symptomatology (2). Members of the transforming growth factor (TGF) β family (activin,

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myostatin, crypto), which play a crucial role in the development of fibrosis (3–5), are highly expressed in endometriotic lesions (6–13). TGFβ1, initially synthesized by monocytes and lymphocytes recruited at the site of inflammation, induces a profibrogenic phenotype with the generation of extracellular matrix by myofibroblasts, non-muscle contractile cells activated in response to injury. Different mechanisms have been proposed to explain the presence of myofibroblasts at the level of endometriotic lesions, such as epithelial-to-mesenchymal transition (EMT) and resident fibroblast transdifferentiation into myofibroblast (14).

Recently, Zhang et al. showed that in endometriosis activated platelets induce EMT, fibroblast-to-myofibroblast transdifferentiation, and differentiation into smooth muscle cells, leading to fibrosis via the TGF-β/Smad signaling pathway (15). Interestingly, the inoculation of human endometrium into a nude mouse model induced alpha smooth muscle actin (α-SMA) expression in murine fibroblasts that surround the lesion and not in human endometrial stroma, suggesting that local environment reacts to the presence of ectopic endometrium (16).

One of the genes modulated by TGFβ is *sphingosine kinase 1* (*SPHK1*), coding the enzyme responsible for the generation of the bioactive sphingolipid sphingosine 1-phosphate (S1P) (17). S1P is a pleiotropic molecule involved in the regulation of inflammation, immune response, angiogenesis, and tumorigenesis (17, 18). The sphingolipid is generated by the adenosine triphosphate–dependent phosphorylation of sphingosine catalyzed by two different isoforms of sphingosine kinase (SK), SK1 and SK2, and it can be converted back to sphingosine by the action of specific S1P phosphatases (SPP), SPP1 and SPP2, or irreversibly catabolized by S1P lyase (SPL) to hexadecenal and phosphoethanolamine. The majority of S1P effects depends on the binding to its specific receptors, named S1P receptors (S1PR) after its release in the extracellular medium by transporters such as spinster homologue 2 (Spns2). Five different isoforms of S1PR have been identified, $S1P_{1-5}$, which couple to different G-proteins and mediate the activation of multiple downstream signaling pathways (19). Moreover, extensive cross-talk between S1P and the TGFβ/Smad signaling cascade has been widely reported (20). The balance between SK1 and SPL expression is crucial for the development of fibrosis: whereas lack of SK1 protects mice against pulmonary fibrosis, SPL deficiency worsens the fibrogenetic process (21).

Recently, it has been shown that stromal endometrial cells from women affected by endometriosis are resistant to apoptosis after exposure to sphingosine analogues, highlighting a dysregulated sphingolipid apoptotic signaling in these cells (22). A possible role of sphingolipids in inducing increased viability of endometriotic cells is suggested by the findings that the expression of the enzymes of S1P metabolism is altered in endometriotic lesions in favor of a decreased catabolism (23).

In the present study, the expression levels of the enzymes involved in S1P metabolism, S1PR, specific S1P transporter Spns2, and SK1-modulating protein calcium- and integrin-binding protein 1 (CIB1), together with some fibrotic markers, were investigated in the two different forms of endometriotic lesions, OMA and DIE. Moreover, in uterine adenocarcinoma cells, we highlighted the possible molecular mechanism by which S1P mediates the profibrotic action of TGFβ, characterizing the S1PR isoforms involved.

MATERIALS AND METHODS

Patients and Tissue Collection

Tissue samples were collected from patients with endometriosis undergoing laparoscopic surgery at Careggi University Hospital, Florence, Italy. Endometriosis samples were categorized as: OMA (n = 15) or DIE (n = 30). Endometriosis was scored according to the revised American Fertility Society classification. Control endometrial specimens were collected during diagnostic hysteroscopy during the proliferative phase from 30 nonpregnant women not affected by endometriosis or other uterine disorders. The clinical and imaging investigations excluded endometriosis and other uterine disorders. All samples were histologically characterized. The endometrial cycle phase was confirmed by histologic analysis of endometrial biopsies. There was no difference in age, gravidity, and parity between the study and control groups. The patients stopped hormonal treatment at least 3 months before surgery. The institutional review board (protocol no. 13742) approved the study protocol, and all patients gave informed written consent.

Materials

All biochemical and cell culture reagents, RPMI 1640, fetal bovine serum (FBS), protease inhibitor cocktail, α-SMA antibody, and bovine serum albumin (BSA) were purchased from Merck Millipore. Short interfering RNAs (siRNAs) were from Sigma-Proligo. Recombinant TGFβ1 was obtained from PeproTech. The human-specific TaqMan Gene Expression Assays used for gene expression studies were purchased from Thermo Fisher Scientific. Anti–SM22-alpha antibody was from Everest Biotech. Secondary antibodies conjugated to horseradish peroxidase, anti-vimentin and anti-β-actin (transgelin) antibodies were obtained from Santa Cruz Biotechnology.

Cell Culture

Uterine adenocarcinoma cells (HeLa), obtained from the American Type Culture Collection, were routinely grown in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2. For the experiments, cells were seeded and, when subconfluent, were serum starved in RPMI 1640 without serum containing 1 mg/mL BSA.

Cell Transfection

Cell transfection was performed with the use of Lipofectamine RNAiMAX according to the manufacturer's instructions as previously reported (24, 25). Briefly, Lipofectamine RNAiMAX was incubated with siRNAs in RPMI 1640 without serum and antibiotics at room temperature for 20 minutes,

and then the lipid/RNA complexes were added with gentle agitation to cells to a final concentration of 50 nmol/L in RPMI 1640 containing serum. After 24 hours, cells were serum starved and used for experiments within 48 hours from the beginning of transfection. The efficacy of specific target down-regulation was evaluated with the use of real-time reverse-transcription polymerase chain reaction (RT-PCR).

Quantitative Real-Time PCR

Total RNA extracted from formalin-fixed and paraffin-embedded tissue sections (500 ng) with the use of the kit All-Prep DNA/RNA FFPE (Quiagen) or from cells (2 μg) with the use of TRI-Reagent was reverse transcribed with the use of a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's protocol as previously described (26, 27). The quantification of target gene mRNAs was performed in triplicate with the use of real-time PCR using TaqMan gene expression assays and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Target sequences were simultaneously amplified together with the housekeeping gene β-actin. Relative quantification of mRNA expression was performed by means of the $2^{-\Delta Ct}$ method (28) for tissue samples or the $2^{-\Delta\Delta Ct}$ method (29) for cellular samples.

Western Blot Analysis

To prepare total cell lysates, cells were incubated for 30 minutes at 4°C in 50 mmol/L Tris, pH 7.5, 120 mmol/L NaCl, 6 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 15 mmol/L $Na_4P_2O_7$, 1% Nonidet, and protease inhibitor cocktail (1.04 mmol/L 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride, 0.02 mmol/L leupeptin, 0.08 μmol/L aprotinin, 15 μmol/L pepstatin A, 0.04 mmol/L bestatin, and 14 μmol/L E-64) before being centrifuged for 15 minutes at 10,000*g* at 4°C accordingly to previously described methods (30, 31). Samples resuspended in Laemmli sodium dodecyl sulphate (SDS) sample buffer were subjected to SDS–polyacrylamide gel electrophoresis before transfer of proteins to polyvinylidene difluoride membranes. Membranes were incubated overnight with the primary antibodies at 4°C and then with secondary antibodies for 1 hour at room temperature. Chemiluminescence was used to detect bound antibodies.

Statistical Analysis

To perform densitometric analysis of the Western blot bands and graphical representations, ImageJ software and GraphPad Prism 6.0 were used, respectively. Statistical analysis was performed with the use of Student *t* test, one-way analysis of variance (ANOVA), and 2-way ANOVA followed by Bonferroni post hoc test.

RESULTS

The mRNA expression of the enzymes involved in S1P metabolism showed that SK1 mRNA levels were significantly higher in OMA ($P<.01$) and DIE ($P<.01$) than in control samples and that the expression levels of SK1 mRNA were not significantly different between OMA and DIE (Fig. 1). Interestingly, the expression level of CIB1, which acts as SK1-interacting protein required for its membrane translocation (32), also was higher in OMA ($P<.0001$) and DIE ($P<.05$) than in control samples (Fig. 1). In contrast, the expression of the other isoform, SK2, was not different in endometriosis samples. Regarding the enzyme involved in the irreversible degradation of S1P, SPL expression was decreased in DIE ($P<.05$) and increased in OMA ($P<.0001$) compared wth control samples (Fig. 1).

We next analyzed the expression levels of S1PRs in OMA and DIE (Fig. 2). The receptors were expressed in healthy endometrium to different extents ($S1P_3 >> S1P_1 >> S1P_4 > S1P_2 >> S1P_5$) and deeply remodeled in pathologic samples (OMA: $S1P_3 >> S1P_5 > S1P_1 >> S1P_4 > S1P_2$; DIE: $S1P_3 >> S1P_5 > S1P_1 >> S1P_4 \approx S1P_2$; Fig. 2A). Although $S1P_1$ was transcriptionally more expressed in OMA ($P<.01$), $S1P_3$ and $S1P_5$ mRNA levels were significantly augmented in both OMA ($P<.0001$ for $S1P_3$ and $P<.05$ for $S1P_5$) and DIE ($P<.05$ for $S1P_3$ and $S1P_5$; Fig. 2B). The mRNA expression of Spns2, the specific transporter involved in the extracellular release of S1P, was higher in DIE ($P<.05$) than in healthy endometrium (Fig. 2B).

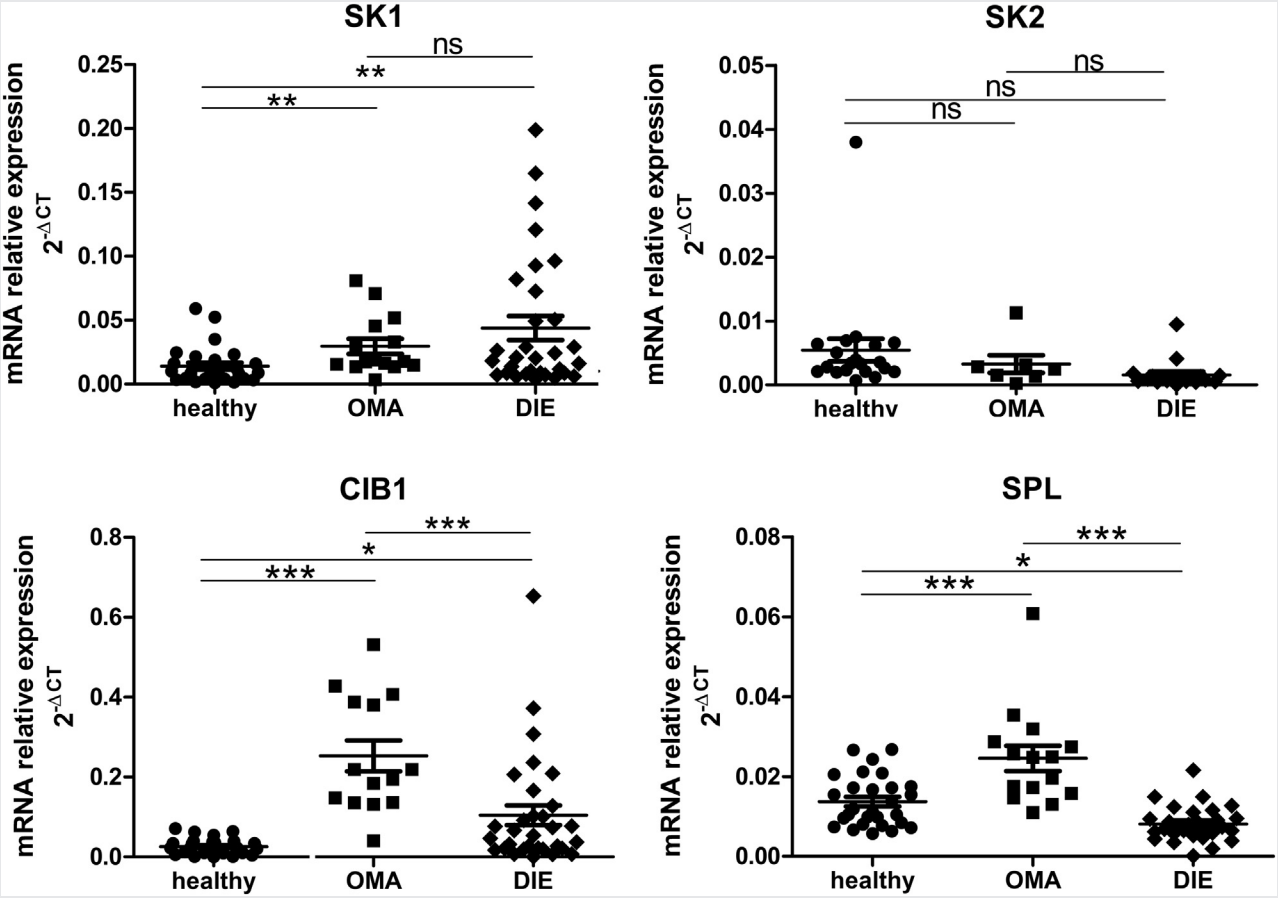
In view of the pivotal role exerted by TGFβ in the onset of fibrotic tissue (3, 4) and the crosstalk between TGFβ and S1P (20), we also investigated the mRNA expression levels of TGFβ1, α-SMA, and collagen type I (COL1A1) in endometriotic lesions, they were significantly higher in OMA ($P<.01$ for TGFβ1, $P<.05$ for α-SMA, and $P<.0001$ for COL1A1) and DIE ($P<.05$ for TGFβ1, α-SMA, and COL1A1) than in healthy endometrium (Supplemental Fig. 1, available online at www.fertstert.org).

To further investigate whether TGFβ1 modulates the expression levels of molecules involved in S1P signaling, uterine adenocarcinoma cells were treated for 6 hours with TGFβ1 (5 ng/mL): the mRNA expression levels of SK1 and SPL were significantly increased ($P<.05$ for SK1 and SPL) while those of SK2 and SPP1 were not altered (Fig. 3A). TGFβ1 also induced an up-regulation of $S1P_1$ ($P<.05$), $S1P_3$ ($P<.05$), and $S1P_5$ ($P<.05$) mRNA levels (Fig. 3B).

The profibrotic role of TGFβ1 was confirmed by the evidence of an increased expression of fibrotic and EMT markers α-SMA ($P<.05$), transgelin ($P<.05$), and vimentin ($P<.05$) according to Western blot analysis (Supplemental Fig. 2A [available online at www.fertstert.org]; Fig. 3C) and increased mRNA level of connective tissue growth factor (CTGF; $P<.05$) (Supplemental Fig. 2B) in our *in vitro* model of EMT.

To investigate the role of S1P signaling and metabolism in the effect of TGFβ1, cells were transfected with specific siRNA that effectively down-regulated the expression of $S1P_1$ ($P<.01$), $S1P_2$ ($P<.01$), $S1P_3$ ($P<.01$), SK1 ($P<.01$), and SK2 ($P<.01$; Supplemental Fig. 3, available online at www.fertstert.org). As shown in Figure 3C, TGFβ1-induced increase of vimentin, α-SMA, and transgelin was significantly reduced in cells where $S1P_2$ ($P<.05$), $S1P_3$ ($P<.05$), SK1 ($P<.05$), and SK2 ($P<.05$) were specifically down-regulated, highlighting that SK1/SK2 activation and $S1P_2$/$S1P_3$ engagement are required to mediate TGFβ1 profibrotic

FIGURE 1



Sphingosine 1-phosphate (S1P) metabolism is dysregulated in endometriosis. Real-time polymerase chain reaction analysis was performed with the use of TaqMan Gene Expression Assay probes specific for S1P enzymes SK1, SK2, SPL, and SK1-modulating protein CIB1 in healthy endometrium (healthy; n = 30), endometrioma (OMA; n = 15), and deep infiltrating endometriotic lesions (DIE; n = 30). Results were analyzed with the use of the $2^{-\Delta Ct}$ method. Statistical differences were tested by means of one-way analysis of variance followed by Bonferroni post hoc test: *$P<.05$; **$P<.01$; ***$P<.0001$; ns = not significant.

Bernacchioni. S1PR dysregulation in endometriosis. Fertil Steril 2020.

action in these cells. These results support the notion that the S1P signaling axis plays a critical role in TGFβ-induced appearance of fibrotic phenotype and EMT.

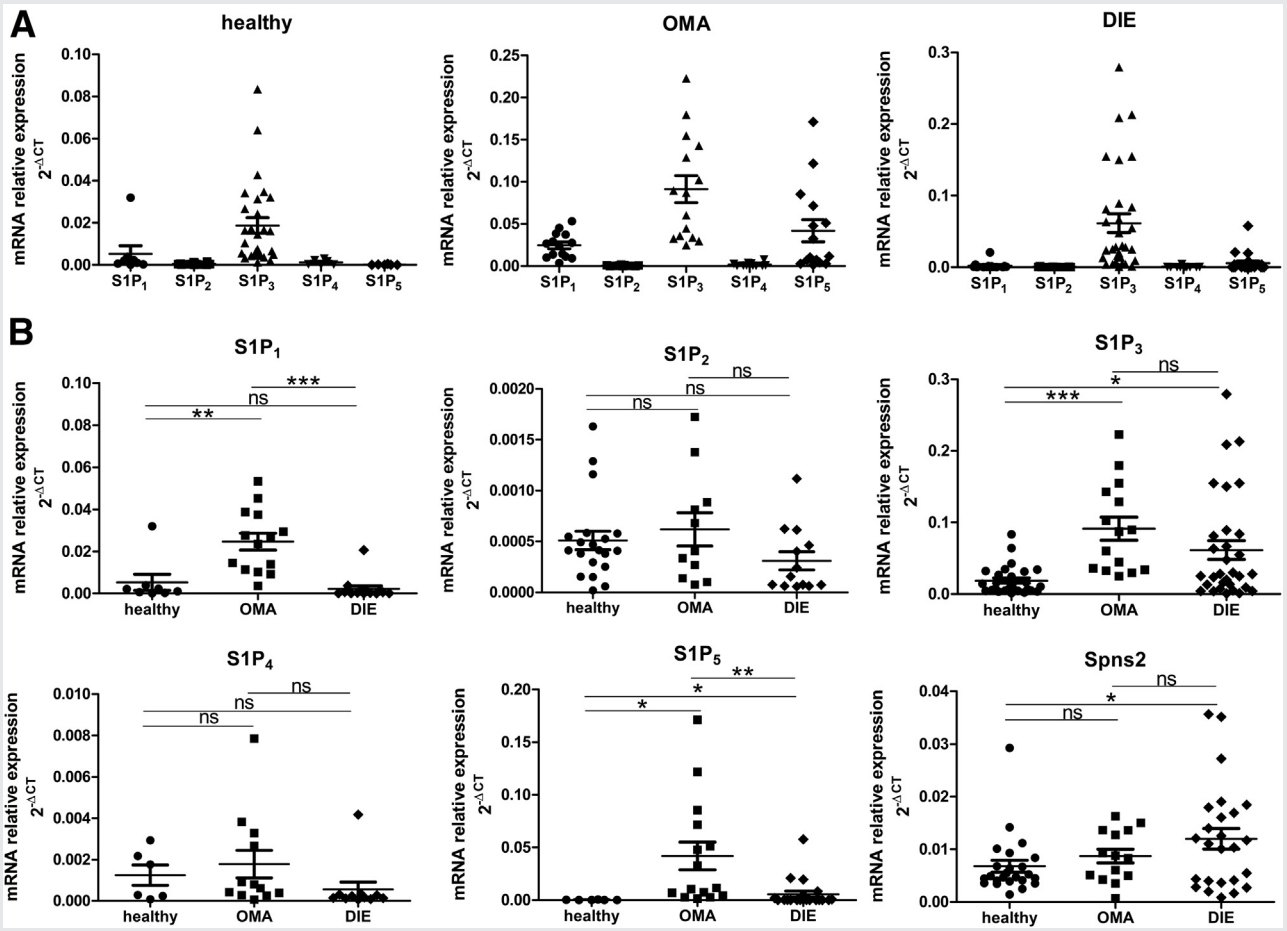
Finally, the profibrotic role of S1P was investigated (Fig. 4). The sphingolipid significantly increased the protein expression levels of vimentin ($P<.05$), α-SMA ($P<.05$), and transgelin ($P<.05$; Fig. 4A) and CTGF mRNA expression ($P<.05$; Fig. 4B). To study the involvement of S1PR in the observed action exerted by S1P, cells were transfected with siRNAs specific for $S1P_1$, $S1P_2$, or $S1P_3$ before being challenged with S1P. When $S1P_1$, $S1P_2$, and $S1P_3$ were down-regulated, the ability of S1P of increasing the fibrotic markers was significantly reduced ($P<.05$ for $S1P_1$, $S1P_2$, and $S1P_3$ down-regulation; Fig. 4C).

DISCUSSION

Altered endometrial cell migration, proliferation, survival, and neoangiogenesis have been implicated in the development of endometriosis (1, 33). Fibrosis is also a constant feature of endometriotic lesions, contributing to pain and infertility, and the molecular mechanisms involved in the appearance of the fibrotic trait in endometriosis are under investigation (5, 16, 26). The findings generated in the present study, showing the role of S1P signaling in the onset of fibrosis, the remodeling of S1PR, and the dysregulation of S1P metabolism in endometriosis, support the hypothesis that the profibrotic role of TGFβ1 in endometriosis is mediated by the S1P pathway, thus involving the bioactive sphingolipid in the pathophysiology of the disease.

S1PRs play multiple and different roles in most physiologic processes and in many disease states, including inflammation and cancer (18, 19). In the present work, we demonstrated that S1PRs are highly expressed in endometriotic lesions. In OMA and DIE, $S1P_5$ became the second most expressed receptor after $S1P_3$, the most expressed receptor in endometriotic lesions, whose mRNA levels are also significantly increased. Whereas $S1P_1$, $S1P_2$, and $S1P_3$ are almost ubiquitously expressed, the expression of $S1P_5$, similarly to that of $S1P_4$, is restricted to distinct cell types, such as nervous

FIGURE 2



Sphingosine 1-phosphate (S1P) signaling is dysregulated in endometriosis. Real-time polymerase chain reaction analysis was performed with the use of TaqMan Gene Expression Assay probes specific for $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$, and Spns2 in healthy endometrium (healthy; n = 30), endometrioma (OMA; n = 15), and deep infiltrating endometriotic lesions (DIE; n = 30). (**A**) Data relative to $S1P_{1-5}$ are reported to highlight S1P receptors relative expression in healthy endometrium, OMA, and DIE. (**B**) Data are reported for individual S1P receptors and S1P-specific transporter Spns2 to compare the expression levels in healthy endometrium vs. OMA vs. DIE. Results were analyzed with the use of the $2^{-\Delta Ct}$ method. Statistical differences were tested by means of one-way analysis of variance followed by Bonferroni post hoc test: *P<.05; **P<.01; ***P<.0001; ns = not significant.

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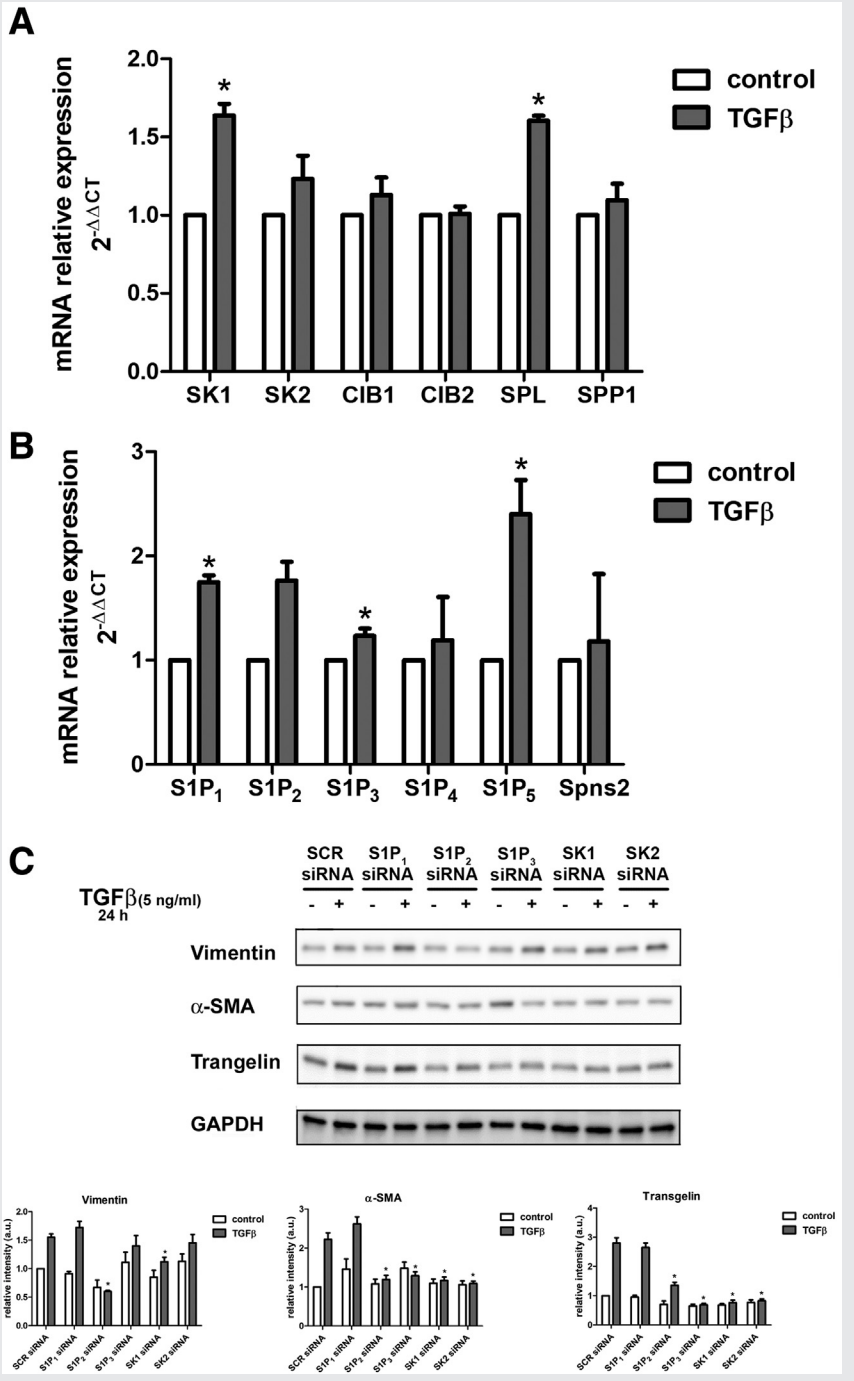
system and lymphatic cells, respectively (19). Therefore, the observed increased levels of $S1P_5$ in endometriosis is compatible with the process of neurogenesis in OMA and DIE associated with pelvic pain in these patients (2, 34).

We also reported that the mRNA levels of $S1P_1$ are very high in OMA samples. This observation might suggest that this receptor could mediate the increased cell migration, proliferation, and survival observed in this form of endometriosis, as previously reported in other tissues (35–37), and might support some difference between OMA and DIE lesions (2). In addition, $S1P_1$ has been involved in nociceptive processing; indeed, the well characterized $S1P_1$ antagonist W146 attenuated peripheral sensitization and thermal hyperalgesia in rat models (38). Further studies are required to dissect the exact role of $S1P_1$ and $S1P_5$ in nociceptive responses in endometriotic lesions.

Anyhow, the increased $S1P_3$ levels in endometriotic lesions are a hallmark of fibrosis, and our *in vitro* data demonstrated that TGFβ is able to remodel S1PR expression, up-regulating mRNA levels of $S1P_3$. This receptor is involved in the onset of fibrosis in different tissues, such as skeletal muscle (39, 40), lung (41), kidney (42), and heart (43), while $S1P_3$ deficiency alleviates radiation-induced pulmonary fibrosis (44). We previously showed that in skeletal muscle myoblasts, after TGFβ1 stimulation $S1P_3$ becomes the most expressed receptor and SK1 is up-regulated and that the SK1/$S1P_3$ signaling axis mediates the transdifferentiation of myoblasts into myofibroblasts induced by TGFβ1 (40). The present data support the hypothesis that in endometriosis the chronic inflammatory condition associated with elevated levels of TGFβ1 and related growth factors (6–13) could remodel the metabolism and signaling of S1P, supporting a role of the

FIGURE 3



Transforming growth factor (TGF) β1 fibrotic action relies on sphingosine 1-phosphate (S1P) signaling. Quantitative mRNA analysis was performed by means of real-time polymerase chain reaction in total RNA extracted from uterine adenocarcinoma epithelial cells stimulated or not with 5 ng/mL TGFβ1 for 6 hours. The mRNA quantification of (**A**) S1P metabolism enzymes (SK1, SK2, SPL, and SPP1) and SK1-modulating proteins (CIB1 and CIB2) and (**B**) S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$) and S1P-specific transporter Spns2 was based on the $2^{-\Delta\Delta Ct}$ method, using individual enzyme, S1PR, Spns2, or SK1-modulating protein of the unchallenged specimen as calibrator. Data are shown as mean ± SEM of three independent experiments performed in triplicate. TGFβ1 increases the expression of SK1, SPL, $S1P_1$, $S1P_3$, and $S1P_5$ in a statistically significant manner (Student *t* test: **P*<.05). (**C**) Cells, transfected with scrambled (SCR), $S1P_1$, $S1P_2$, $S1P_3$, SK1, or SK2 short interfering (si) RNA, were challenged with 5 ng/mL TGFβ1 for the last 24 hours of transfection. Western analysis of fibrotic marker proteins vimentin, alpha smooth muscle actin (α-SMA), and transgelin was performed in cell lysates. Blot representatives of three independent experiments with analogous results are shown. The histograms represent the densitometric analysis of at least three independent experiments. Data are shown as mean ± SEM and are reported as protein expression normalized to GAPDH, fold change over control. The effect of $S1P_2$, $S1P_3$, SK1, and SK2 down-regulation on TGFβ1 fibrotic effect was tested for statistical significance by means of two-way analysis of variance followed by Bonferroni post hoc test: **P*<.05.

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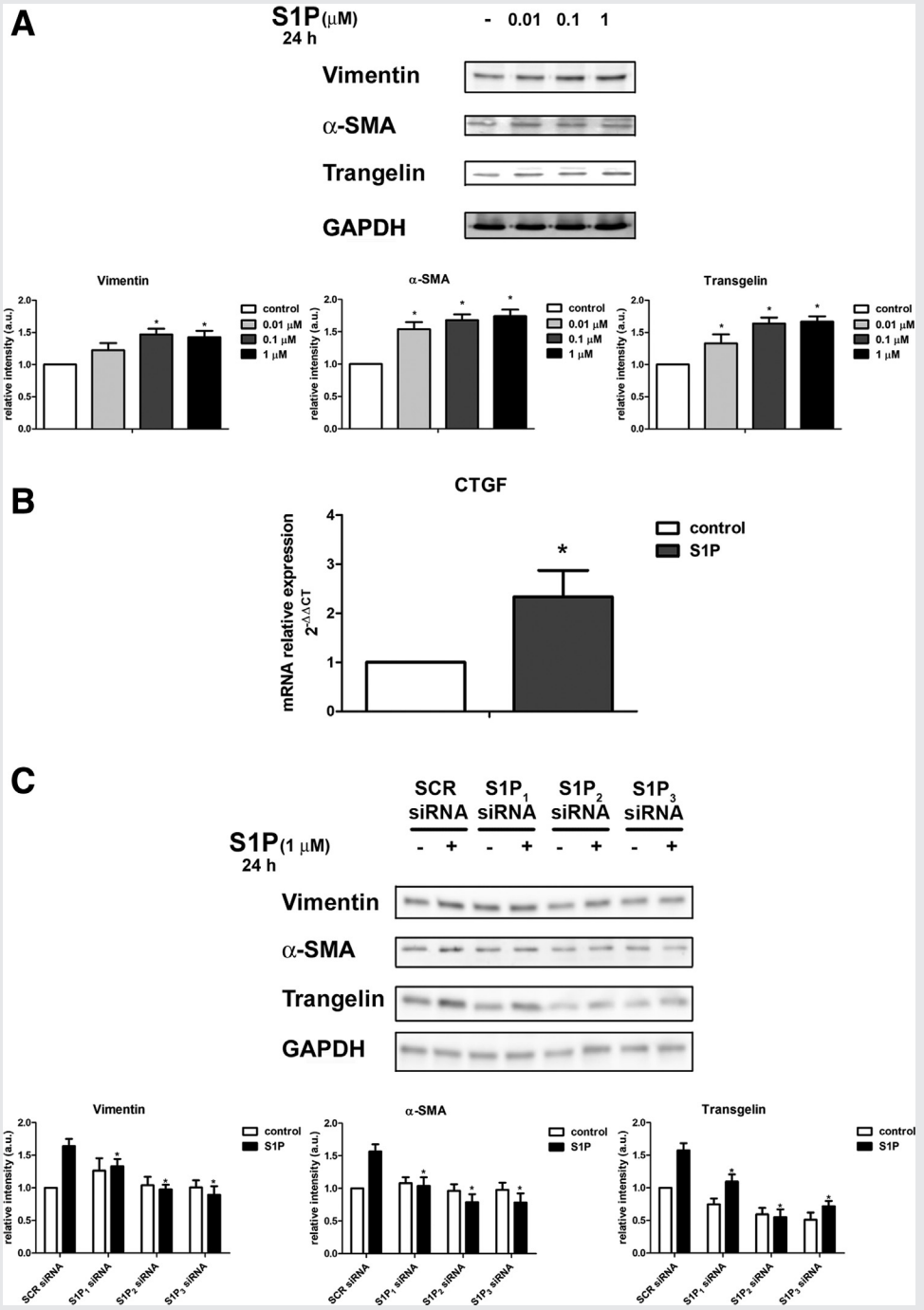
FIGURE 4



Sphingosine 1-phosphate (S1P) exerts a profibrotic action. (**A**) Uterine adenocarcinoma cells were treated with different S1P concentrations (0.01, 0.1, and 1 μmol/L) for 24 hours. The content of vimentin, alpha smooth muscle actin (α-SMA), and transgelin was analyzed by means of Western blotting of whole cell lysates. Blot representatives of three independent experiments with analogous results are shown. The histograms represent the densitometric analysis of at least three independent experiments. Data are shown as mean ± SEM and are reported as protein expression normalized to GAPDH, fold change over control. S1P significantly increased the expression of fibrosis proteins (one-way analysis of variance followed by Bonferroni post hoc test: *P<.05). (**B**) Quantitative mRNA analysis of connective tissue growth factor (CTGF) was performed by means of real-time polymerase chain reaction in total RNA extracted from cells stimulated or not with 1 μmol/L S1P for 6 hours. S1P increased the expression of CTGF in a statistically significant manner (Student t test: *P<.05). (**C**) Cells, transfected with scrambled (SCR) $S1P_1$, $S1P_2$, $S1P_3$, SK1, or SK2 short interfering (si) RNA, were treated with 1 μmol/L S1P for the last 24 hours of transfection. Western analysis of fibrotic marker proteins was performed in cell lysates. Blot representatives of three independent experiments with analogous results are shown. The histograms represent the densitometric analysis of at least three independent experiments. Data are shown as mean ± SEM and are reported as protein expression normalized to GAPDH, −fold change over control. The effect of $S1P_1$, $S1P_2$, and $S1P_3$ down-regulation on S1P-induced fibrotic effect was tested for statistical significance by means of two-way analysis of variance followed by Bonferroni post hoc test: *P<.05.

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sphingolipid in the pathogenesis of the disease. TGFβ1 modulates the transcripts of genes codifying for enzymes involved in S1P metabolism as well as S1PR: TGFβ1 up-regulated SK and $S1P_3$ mRNA levels in uterine adenocarcinoma cells, and its profibrotic effect was abolished when SKs or $S1P_{2/3}$ were blocked, suggesting that the bioactive sphingolipid mediates the profibrotic effect of TGFβ1. The present study showed for the first time that both OMA and DIE lesions have increased SK1 and $S1P_3$, while the pronounced decreased SPL expression in DIE suggests some differences in the biology of these lesions (2). The net effect resulting from such deregulation leads to the increase of S1P signaling. A significant increase of α-SMA, TGFβ1, and COL1A1 mRNA levels was here reported in both types of endometriotic lesions, in agreement with immunostaining data on the protein increase of α-SMA and other EMT/fibrosis markers in OMA and DIE (45). These data fit with the evidence that endometriotic lesions, stimulated by TGFβ1, activate the Smad3 signaling pathway and undergo EMT, ultimately resulting in fibrosis (15). Interestingly, platelets release TGFβ1 on activation (46) and drive EMT and fibroblast-to-myofibroblast transition in endometriosis (15). Of note, platelets release large amounts of S1P because they do not express the enzymes responsible for S1P degradation (47), suggesting that the sphingolipid might be involved in a platelet-induced effect in endometriosis. In this regard, an original finding of the present study is the demonstrated ability of S1P itself of increasing the expression levels of fibrosis markers via S1PRs.

Interestingly, the increase of SK1 is mirrored by more elevated mRNA levels of CIB1, a ubiquitous protein involved in enhancing cell survival, proliferation, and tumor angiogenesis that has been shown to modulate the subcellular localization of SK1 and its membrane translocation, thus controlling S1P cellular generation (32, 48). Indeed, it has been previously demonstrated that overexpression of CIB1 by itself is sufficient to drive localization of SK1 to the plasma membrane and enhance the membrane-associated enzymatic activity of SK1 and its oncogenic signaling (49).

In addition to the alteration of the expression of S1PRs and the enzymes involved in S1P metabolism, we showed that the expression levels of the specific S1P transporter Spns2, responsible for the extracellular release of the sphingolipid, is altered only in DIE, further highlighting the complex dysregulation of S1P signaling in endometriosis. It will be of great interest in future studies measuring sphingolipid levels in all of the different lesions of endometriosis by means of liquid chromatography–mass spectrometry. Although SK1 mRNA levels did not change between OMA and DIE, those of SPL, which irreversibly degrades S1P, and CIB1 significantly diverge, such that different levels of the sphingolipid between the two forms of endometriosis are highly suspected.

The role of bioactive sphingolipids in endometriosis is also supported by altered levels of sphingomyelin in the peritoneal fluid of OMA patients (50), and stromal endometrial cells from women with endometriosis show an increased survival resistant to ceramide-induced apoptosis (22). Gene-expression profiling for OMA highlighted dysregulated expression of genes involved in sphingolipid metabolism (51), with an up-regulation of alkaline sphingomyelinase, ceramidase and SK1 and a down-regulation of SPP1. Santulli et al. reported the down-regulation of SPL in endometriotic tissue (23). Recently, S1P levels were found to be elevated in the cystic fluid of endometriomas and the sphingolipid was able to induce proliferation and interleukin-6 secretion in endometrial stromal cells, supporting its involvement in inflammation and growth of endometriotic cells (52).

The present study also highlights S1PRs as possible endometriosis pharmacologic targets, providing plausible further application for FTY720 (fingolimod)–based therapies. FTY720, a sphingosine analogue that after in vivo phosphorylation acts on all S1PRs except $S1P_2$, is the first orally available agent approved by the U.S. Food and Drug Administration for the treatment of relapsing-remitting multiple sclerosis (53). Inhibition of the S1P axis by FTY720 has been shown to reduce inflammation and produce anticancer effects (54).

CONCLUSION

The present data showed that the signaling and the metabolism of the bioactive lipid S1P are profoundly altered in endometriosis. Indeed, for the first time, a marked remodeling of S1PR expression was highlighted in both OMA and DIE. Moreover, our findings demonstrate that S1P mediates the ability of TGFβ1 to induce fibrosis and EMT markers in an *in vitro* EMT model of uterine adenocarcinoma cells. Using RNA interference approaches we showed that SKs and $S1P_2$/$S1P_3$ are involved in this action.

Altogether, our findings support the view that the S1P signaling axis may be a biomarker for endometriosis and useful for diagnostic and therapeutic purposes.

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Los receptores de la Esfingosina 1-fosfato están alterados en endometriosis: Posible implicación en la fibrosis inducida por el factor de crecimiento transformador beta.

Objetivo: Estudiar los mecanismos moleculares implicados en la característica de apariencia fibrótica en endometriosis mediante la investigación de si la vía de señalización del esfingolípido Esfingosina 1-fosfato (SP1) bioactivo estaba alterada en las lesiones endometriosicas.

Diseño: Un estudio caso-control de laboratorio.

Lugar: Instituto de investigación universitario y hospital universitario.

Paciente(s): Un total de 75 mujeres, con y sin endometriosis, fueron incluidas en el estudio.

Intervención: Muestras endometriales fueron obtenidas a partir de mujeres afectadas (n=15 endometrioma [OMA]; n=30 endometriosis profunda infiltrante [DIE]) y no afectadas (n=30) por endometriosis por medio de una operación por laparoscopia, seguida de una investigación clínica y por imagen, así como comprobar la expresión de marcadores de fibrosis y genes implicados en el metabolismo y señalización de SP1 por medio de la reacción en cadena de la polimerasa a tiempo real.

Medida(s) principales: El papel del eje de señalización SP1 en la fibrosis asociada a endometriosis fue estudiado in vitro, donde se usaron aproximaciones de RNA de interferencia para investigar si la síntesis de SP1 por las esfingosinas kinasas (SKs) y receptores específicos de SP1 (S1PRs) están implicados en el efecto profibrótico de la citoquina factor de crecimiento transformador beta 1.

Resultado(s): El análisis de la expresión del ARNm de S1PR demostró una alteración profunda de la señalización de SP1 en endometriosis, caracterizada por un aumento de expresión en los marcadores de fibrosis: $S1P_1$ estuvo transcripcionalmente más expresado en OMA, y los niveles de ARNm de $S1P_3$ y $S1P_5$ estuvieron aumentados significativamente en OMA y DIE. SK1 y su proteína activadora de unión 1 al calcio y a la integrina (CIB1) estuvieron significativamente reguladas al alza en OMA y DIE. Un papel crucial del eje SK/S1PR en el efecto profibrótico mediado por TGFb1 fue resaltado in vitro.

Conclusión(es): El eje de señalización SP1 podría representar un biomarcador útil o como diana farmacológica innovadora para la endometriosis.

Sphingosine 1-phosphate signaling in uterine fibroids: implication in activin A pro-fibrotic effect

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Objective: To explore the link between sphingosine 1-phosphate (S1P) signaling and leiomyoma and the possible S1P cross-talk with the fibrotic effect of activin A.
Design: Case-control laboratory study.
Setting: University institute and university hospital.
Patient(s): Patients with uterine fibroids (n = 26).
Interventions(s): Tissue specimens of leiomyoma and normal myometrium were obtained from patients undergoing myomectomy or total hysterectomy.
Main Outcome Measure(s): Expression of mRNA levels of the enzyme involved in S1P metabolism, S1P receptors, and S1P transporter Spns2 was evaluated in matched leiomyoma/myometrium specimens and cell populations. The effects of inhibition of S1P metabolism and signaling was evaluated on activin A–induced fibrotic action in leiomyoma cell lines.
Result(s): The expression of the enzymes responsible for S1P formation, sphingosine kinase (SK) 1 and 2, and $S1P_2$, $S1P_3$, and $S1P_5$ receptors was significantly augmented in leiomyomas compared with adjacent myometrium. In leiomyoma cells, but not in myometrial cells, activin A increased mRNA expression levels of SK1, SK2, and $S1P_2$. The profibrotic action of activin A was abolished when SK1/2 were inhibited or $S1P_{2/3}$ were blocked. Finally, S1P augmented by itself mRNA levels of fibrotic markers (fibronectin, collagen 1A1) and activin A in leiomyomas but not in myometrial cells.
Conclusion(s): This study shows that S1P signaling is dysregulated in uterine fibroids and involved in activin A–induced fibrosis, opening new perspectives for uterine fibroid treatment. (Fertil Steril® 2021;115:1576–85. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Uterine fibroids, leiomyomas, sphingosine 1-phosphate, sphingosine 1-phosphate receptors, sphingosine kinase, fibrosis, activin A

Uterine leiomyomas, or uterine fibroids, represent the most common benign gynecologic tumors causing significant morbidity and affecting reproductive function with an impact on fertility and pregnancy outcome (1, 2). Various hormones, such as sex steroid hormones, as well as epigenetic and genetic signatures influence the pathogenesis of uterine fibroids (3). Despite the high prevalence and clinical impact, the pathogenesis is still debated. Uterine fibroids are characterized by increased expression of extracellular matrix (ECM), collagen, fibronectin, and proteoglycans and elevated levels of inflammatory mediators (cytokines and chemokines) (4, 5), so it is also defined as a fibrotic disease (6, 7). Uterine fibroids originate from an abnormal activation of myofibroblasts and stem cells, followed by an improper fibrinogenesis and inflammatory response (3). The overproduction of ECM causes rigidity of the structure, causing symptoms such as abnormal bleeding, pelvic

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pressure, and pain (8, 9). A critical role in the formation and growth of uterine fibroids is played by gonadal sex steroid hormones (estrogens, progesterone), whose action is mediated by local production of growth factors (5, 10, 11).

Activin A, belonging to transforming growth factor (TGF) β superfamily, is highly expressed in leiomyoma tissue compared with the adjacent myometrial tissues (10, 12); interestingly, activin A treatment induced ECM protein expression in leiomyoma cells, suggesting a key role in the profibrotic process of uterine fibroids (13), as also supported by the evidence that ulipristal acetate, a hormonal drug used for the fibroid treatment, reduces activin A expression in vitro (14). The evidence that tissues surrounding the leiomyoma show increased recruitment of macrophages suggests activation of the inflammatory process (15, 16). Interestingly, while tumor necrosis factor (TNF) α increases the expression of activin A in myometrial and leiomyoma cells (15), treatment with the antinflammatory drug tranilast reduces activin A as well as ECM expression in both cell types (17).

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid crucially involved in the modulation of multiple biological processes, such as proliferation, differentiation, migration, and survival (18, 19). Therefore, sphingolipids are studied as potential novel biomarkers for autoimmune or inflammatory disorders and benign or malignant tumors (20). In this regard, modulation of S1P signaling may represent a possible novel therapeutic target (21).

S1P metabolism is finely regulated: Two isoforms of sphingosine kinase (SK), SK1 and SK2, are responsible for its generation via the adenosine triphosphate (ATP)–dependent phosphorylation of sphingosine, whereas specific S1P phosphatases and nonspecific lipid phosphate phosphatases convert S1P back to sphingosine. Alternatively, S1P can be irreversibly cleaved by S1P lyase (SPL) to hexadecenal and phosphoethanolamine. S1P evokes its pleiotropic effects acting both as intracellular messenger and as ligand of five different specific G protein–coupled receptors (S1PRs), named $S1P_{1-5}$ (22), after its release outside the cell through the specific transporter spinster homologue 2 (Spns2) or nonspecific transporters belonging to the ATP-binding cassette (ABC) family.

Increasing experimental evidence demonstrates that S1P, acting as paracrine or autocrine cue, plays an important role in the development of fibrosis of lung (23), liver (24), heart (25), kidney (26), and skeletal muscle (18). In general, S1P promotes fibrosis in a variety of cells, including macrophages, fibroblasts (27, 28), and skeletal muscle precursors (29), by acting in conjunction with other molecules, such as the inflammatory cytokine TNF-α (30), which stimulates the synthesis of interleukin-1β and TGF-β1 in a variety of cells. We previously showed that TGF-β1 induces transdifferentiation of skeletal muscle myoblasts into myofibroblasts via up-regulation of the SK1/$S1P_3$ axis (31). SK1 and $S1P_3$ mRNA expression is also significantly increased in endometriosis, a chronic inflammatory fibrotic disease (32). Moreover, TGF-β1 modulates the transcripts of genes encoding for SK and $S1P_3$ in uterine adenocarcinoma cells, and the profibrotic effect of the cytokine was abolished when SK or $S1P_{2/3}$ was down-regulated (32).

The aim of the present study was to evaluate the S1P pathway expression in uterine fibroids and its possible cross-talk with activin A in modulating the fibrogenetic mechanisms in uterine fibroids.

MATERIAL AND METHODS

Patients and Tissue Collection

A group of premenopausal women (n = 26; age range 33–46 years) from the Department of Gynecology and Obstetrics of Careggi University Hospital (Florence, Italy) were included in the present study. They were admitted for surgical treatment for leiomyomas and underwent myomectomy or total hysterectomy. For each patient, two tissue specimens were collected during surgery: leiomyoma and normal myometrium. Normal uterine smooth muscle cells were used as control samples. Specimens were immediately stored in RNA-later at −80°C until RNA extraction.

For each patient, the demographics (age, body mass index), symptoms (blood loss at menstruations, dysmenorrhea), previous treatment for leiomyoma, infertility, and previous pregnancies were recorded. The local institutional review board approved the research protocol (protocol no. 13742), and every participant signed her informed consent.

Materials

All biochemicals, selective $S1P_2$ antagonist JTE013, specific SK1 inhibitor PF-543, and activin A were purchased from Merck Life Science. Dulbecco Modified Eagle Medium (DMEM), high-glucose DMEM–Ham F-12 and Dulbecco phosphate-buffered saline solution (PBS) were obtained from Corning. Fetal bovine serum (FBS), penicillin-streptomycin solution, amphotericin B, and Hank Balanced Salt Solution (HBSS) were purchased from Euroclone. Selective $S1P_1$ antagonist W146 was from Avanti Polar Lipids (Alabama, USA). Specific $S1P_3$ antagonist, CAY10444, and selective SK2 inhibitor ABC294640 were obtained from Cayman Chemical. Glutamine and human-specific TaqMan gene expression assays used for gene expression studies were purchased from Thermo Fisher Scientific. The AllPrep DNA/RNA/Protein kit was purchased from Qiagen. Bradford protein assay reagent was obtained from Bio-Rad. SK2 (N-terminal region) and SK1 (central region) rabbit polyclonal antibodies were purchased from ECM Biosciences. Anti-$S1P_3$ and anti-$S1P_2$ antibodies were from Bioss Antibodies and Proteintech Europe, respectively. Secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) reagents were obtained from GE Healthcare Europe.

Primary Cell Isolation

After surgery, the myometrial and leiomyoma samples were collected in HBSS and immediately processed. The samples were washed several times with PBS to remove excess blood. After cutting tissue into small pieces, the samples were mixed in 0.1% collagenase type 8 (Serva Electrophoresis) in serum-free DMEM and incubated at 37°C for 3–5 hours in a water

bath with manual shaking. After digestion, the cell suspension was centrifuged at 250*g* for 10 minutes, and the collagenase was inactivated with FBS. Finally, the cell pellet was dispersed in high-glucose DMEM with L-glutamine and sodium pyruvate containing 10% FBS, 1% penicillin-streptomycin, and 1% amphotericin B in T25 plastic dishes, and incubated at 37°C in 95% air, 5% CO_2. The growth medium was changed after 24 or 48 hours to remove unattached cells and subsequently twice a week. The purity of cells was assessed by staining with a specific smooth muscle cell marker (anti–alpha-smooth muscle actin antibody; Merck Life Science). Almost all cells were strongly positive for alpha-smooth muscle actin (data not presented). Cells at the lower passage number (P0–P4) were used for experiments to avoid changes in phenotype and gene expression.

Cell Lines Culture

The myometrial and leiomyoma cell lines were generously provided by William H. Catherino, M.D., Ph.D. (Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland). These cell lines had been obtained from primary myometrial and leiomyoma cells immortalized following the modified protocol of Rhim (33) using human papillomavirus type 16 as previously described by Malik et al. (34). Cells were cultured in high-glucose DMEM–Ham F-12 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% amphotericin B, and 1% glutamine, at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Treatments

For the treatments, myometrial and leiomyoma cell lines were cultured in fresh DMEM–Ham F-12 supplemented with 0.1% fatty acid free bovine serum albumin (Merck Life Science) and 20% delipidated FBS (Fetal Bovine Serum Charcoal Stripped; Merck Life Science). When requested, myometrial and leiomyoma cell lines were pretreated with SK inhibitors (10 μmol/L PF-543 or 1 μmol/L ABC294640) or S1PR-specific antagonists (10 μmol/L W146, 1 μmol/L JTE013, or 5 μM CAY10444) for 30 minutes before being challenged with activin A for 48 hours.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from tissues with the use of the kit AllPrep DNA/RNA/Protein Mini Kit (Qiagen) or from cells with the use of Trizol reagent (Thermo Fisher). Samples were digested with a ribonuclease-free deoxyribonuclease (Promega Corp.), and the RNA was cleaned up and concentrated with the use of the ReliaPrep RNA Cell Miniprep System (Promega Corp.). Total RNA from tissues (500 ng) or from cells (1 μg) was reverse transcribed with the use of a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's protocol as previously described (35, 36). The quantification of target gene mRNAs was performed in triplicate by means of real-time reverse-transcription polymerase chain reaction (PCR) using TaqMan gene expression assays with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Target sequences were simultaneously amplified together with the housekeeping gene β-actin. Relative quantification of mRNA expression was performed using the $2^{-\Delta Ct}$ method (37) or the $2^{-\Delta\Delta Ct}$ method (38).

Western Blot Analysis

Total proteins were extracted from tissues with the use of the AllPrep DNA/RNA/Protein Kit (Qiagen) and resuspended in a buffer containing 8 mol/L urea in accordance with the protocol provided by manufacturer. Protein lysates were then boiled and sonicated for 5 minutes before being centrifuged at 10,000*g* for 15 minutes at 4°C. Finally, ~18 μg protein from total cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently to Western blot (WB) analysis using primary antibodies against SK1, SK2, $S1P_2$, and $S1P_3$, as previously described (39, 40). Membranes were then incubated with secondary antibodies for 1 hour at room temperature. Bound antibodies were revealed by chemiluminescence with the use of ECL reagents.

Normalization is performed by measuring total protein directly on the membrane that is used for WB by means of stain-free technology (41) that uses a proprietary trihalo compound in Mini Protean TGX Stain-Free Gels (BioRad) to enhance the fluorescence of tryptophan amino acids when exposed to ultraviolet light. Band intensity of target proteins was reported as fold increase relative to the respective control set as 1.

Statistical Analysis

Graphical representations were realized with the use of GraphPad Prism 6.0. To perform densitometric analysis of the Western blot bands, ImageJ software was used. Statistical analysis was performed with the use of Student *t* test and two-way analysis of variance followed by Bonferroni post hoc test.

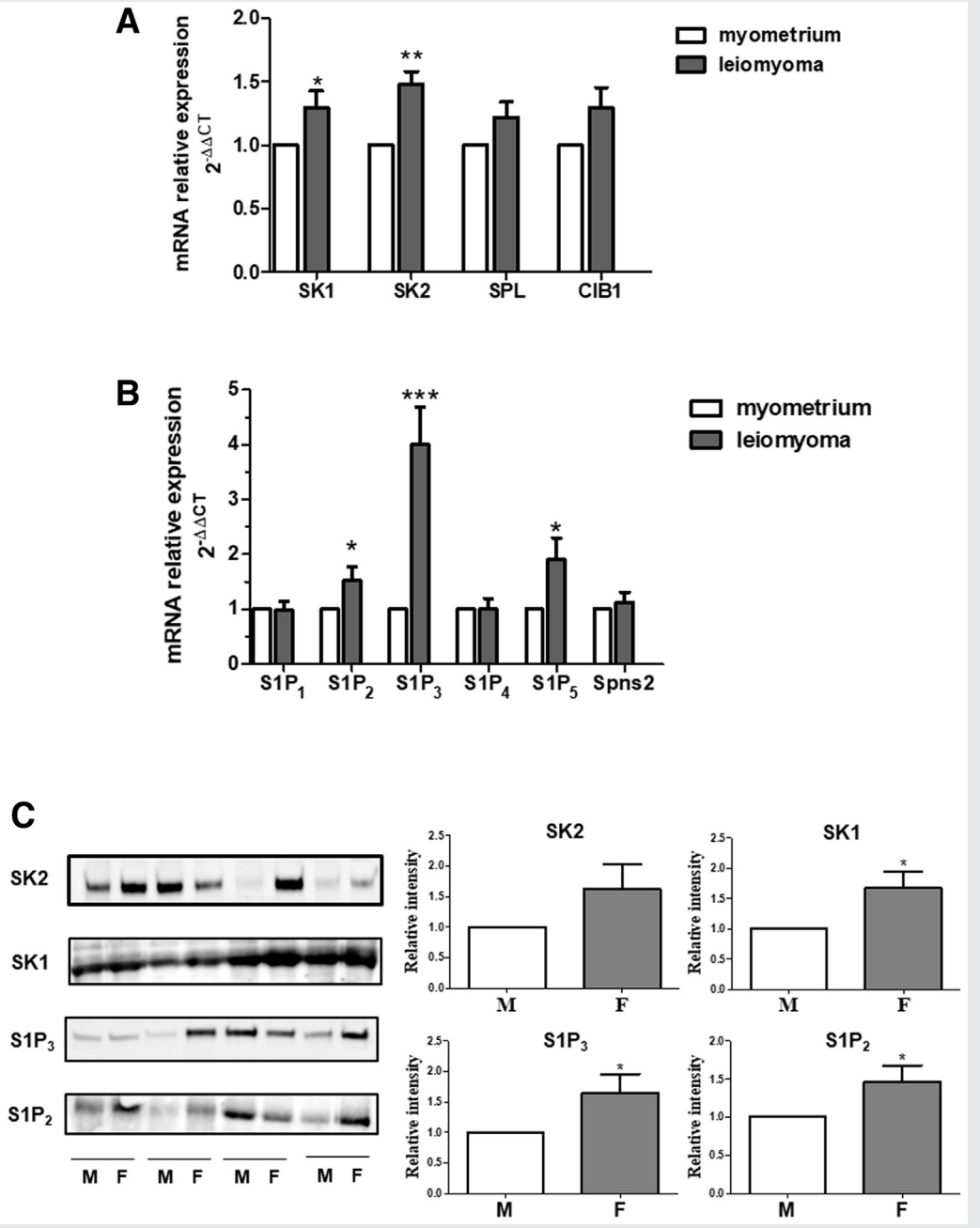
RESULTS

S1P Signaling Dysregulation in Uterine Fibroids

To explore the S1P signaling axis in uterine fibroids, we first examined the expression of enzymes involved in S1P metabolism. Figure 1A shows that SK1 and SK2 mRNA levels were significantly higher in leiomyoma than in adjacent healthy myometrium, whereas the expression levels of SK1-activating protein CIB1 (calcium and integrin-binding protein 1) (42, 43) and SPL, the enzyme catalyzing the irreversible degradation of S1P, were not significantly different between leiomyoma and healthy myometrium.

Real-time PCR analysis of S1PR expression showed that $S1P_2$, $S1P_3$, and $S1P_5$ were highly increased compared with the adjacent healthy myometrium, whereas the mRNA levels of $S1P_1$, $S1P_4$, and Spns2, the specific transporter implicated in the extracellular release of S1P, were not significantly modified (Fig. 1B). Thus, the relative levels of S1PR expression is profoundly altered in leiomyoma compared with the adjacent healthy myometrium, with $S1P_3$ becoming the most expressed receptor (Supplemental Fig. 1 [available online at www.fertstert.org]). To confirm the observed transcriptional changes of the S1P signaling axis, SK1, SK2, $S1P_2$, and

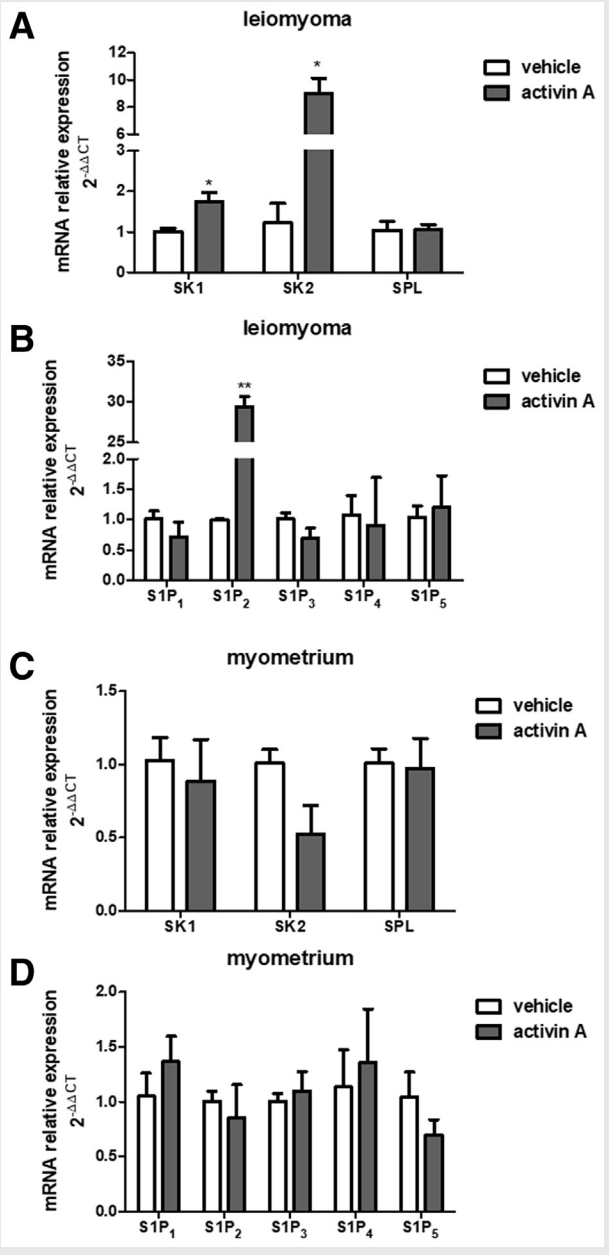
FIGURE 1



S1P metabolism and signaling are dysregulated in uterine fibroids. Real-time polymerase chain reaction analysis was performed using TaqMan Gene Expression Assay probes specific for (**A**) S1P enzymes and SK1-modulating protein CIB1 or (**B**) S1P receptors and S1P-specific transporter Spns2 in uterine fibroids (n = 26) and in the adjacent normal myometrium (n = 26). Results were analyzed with the $2^{-\Delta\Delta Ct}$ method using individual enzyme, CIB1, individual receptor subtype, or Spns2 of the normal myometrium as calibrator. Differences are statistically significant according to Student *t* test: **P*<.05; ***P*<.01; ****P*<.0001. (**C**) Western blot analysis was performed in uterine fibroids (F) and in the adjacent normal myometrium (M) using specific antibodies against SK1, SK2, $S1P_2$, and $S1P_3$. Representative blots are shown. The histograms represent the densitometric analysis of all the analyzed samples (n = 24 F and 24 M for SK1, SK2, and $S1P_3$; n = 11 F and 11 M for $S1P_2$). Data are presented as protein expression (mean ± SEM) normalized on total protein content, as described in Methods. SK1, $S1P_2$, and $S1P_3$ protein levels in uterine fibroid are statistically different from healthy myometrium according to Student *t* test: **P*<.05. S1P = sphingosine 1-phosphate; $S1P_{1-5}$ = S1P receptors 1–5; SK = sphingosine kinase; SPL = S1P lyase.

Bernacchioni. S1P axis in uterine fibroid-associated fibrosis. Fertil Steril 2020.

FIGURE 2



Activin A modulates the S1P signaling axis in leiomyoma cells. Quantitative mRNA analysis was performed with the use of real-time polymerase chain reaction in total RNA extracted from (**A, B**) leiomyoma A006-8 F cells or (**C, D**) myometrial A005-7 M cells stimulated or not with 50 μg/mL activin A for 48 hours. The mRNA quantification of (**A, C**) S1P metabolism enzymes (SK1, SK2, SPL) and (**B, D**) S1PRs was based on the $2^{-\Delta\Delta Ct}$ method, using individual enzyme or S1PR of the unchallenged specimen as calibrator. Data are presented as the mean ± SEM of three independent experiments performed in triplicate. Activin A increases the expression of SK1, SK2, and $S1P_2$ in a statistically significant manner in leiomyoma A006-8 F cells (Student *t* test: **P*<.05; ***P*<.01). S1P = sphingosine 1-phosphate; $S1P_{1-5}$ = S1P receptors 1–5; S1PR = S1P receptor; SK = sphingosine kinase; SPL = S1P lyase.

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$S1P_3$ protein levels were examined by means of WB analysis in uterine fibroids. The findings reported in Figure 1C confirmed the increased expression of SK1, $S1P_2$, and $S1P_3$ in leiomyoma compared with adjacent healthy myometrium.

Activin A Modulates S1P Signaling in Leiomyoma Cells

The possible involvement of the S1P signaling axis in the profibrotic effect of activin A in leiomyoma cells was then investigated. As shown in Figure 2A, activin A deeply modulates S1P signaling in leiomyoma A006-8 F cells. In particular, the treatment with 50 μg/mL activin A for 48 hours strongly up-regulated the mRNA expression of both SK1 and SK2 (~2-fold and ~10-fold increases, respectively). Moreover, quantitative analysis of S1PR expression showed that activin A modulates S1PR mRNA, augmenting $S1P_2$ levels (Fig. 2B). Thus, the S1PR expression profile in untreated leiomyoma A006-8 F cells was $S1P_3 >> SIP_1 > S1P_5 > S1P_4 >> S1P_2$, whereas it became $S1P_3 >> S1P_1 > S1P_2 \geq S1P_5 >> S1P_4$ in activin A–treated cells (Supplemental Fig. 2A [available online at www.fertstert.org]).

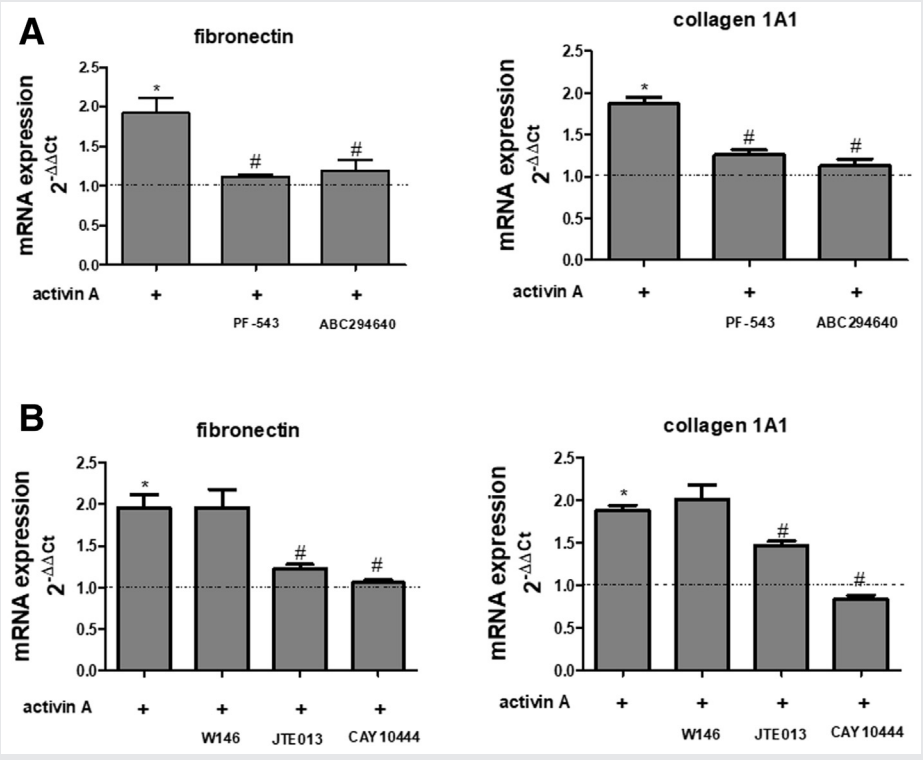
In contrast, in control myometrial A005-7 M cells the treatment with 50 μg/mL activin A for 48 hours did not modulate the expression of S1P metabolism enzymes (Fig. 2C) or the expression of S1PRs (Fig. 2D) (Supplemental Fig. 2B). To validate the results obtained in immortalized cell lines, the effect of activin A was tested in both primary myometrial and leiomyoma cells. Interestingly, the treatment with 50 μg/mL activin A for 48 hours significantly augmented the mRNA expression of SK1, SK2, and $S1P_2$ in leiomyoma primary cells (Supplemental Fig. 3A [available online at www.fertstert.org]) but not in myometrial primary cells (Supplemental Fig. 3B).

The Profibrotic Effect of Activin A in Leiomyoma Cells Is Mediated by S1P Signaling

The potential role of SK1 and SK2 in the profibrotic action of activin A was examined in leiomyoma A006-8 F cells. With this aim, cells were challenged with 50 μg/mL activin A for 48 hours, which strongly augmented mRNA levels of fibrotic markers fibronectin and collagen 1A1 in the presence or absence of 10 μmol/L PF-543, the specific inhibitor of SK1, or 1 μmol/L ABC294640, the selective inhibitor of SK2. Figure 3A shows that activin A profibrotic effect was significantly reduced when SK1 or SK2 was inhibited.

To evaluate whether the profibrotic action exerted by activin A was mediated by S1PRs, the expression of fibrosis markers was examined in leiomyoma A006-8 F cells challenged with activin A in the presence or absence of 10 μmol/L W146, the selective antagonist of $S1P_1$, 1 μmol/L JTE013, which specifically blocks $S1P_2$, or 5 μmol/L CAY10444, which selectively blocks $S1P_3$. As shown in Figure 3B, enhanced mRNA expression of fibronectin and collagen 1A1 induced by 48 hours of treatment with activin A was significantly reduced when $S1P_2$ or $S1P_3$ were blocked

FIGURE 3



Role of S1P (**A**) metabolism and (**B**) signaling in activin A pro-fibrotic effect in leiomyoma cells. (**A**) Leiomyoma A006-8 F cells were pretreated with SK1-specific inhibitor PF-543 (10 μmol/L) or selective SK2 inhibitor ABC294640 (1 μmol/L) for 30 minutes before being challenged with 50 μg/mL activin A for 48 hours. Quantitative mRNA analysis of fibronectin and collagen 1A1 was performed with the use of real-time polymerase chain reaction in total RNA extracted from cells. Activin A increases the expression of fibronectin and collagen 1A1 in a statistically significant manner (Student *t* test: **P*<.05). The effect of SK1 inhibition by PF-543 or SK2 blockade by ABC294640 on activin A profibrotic effect is statistically significant according to two-way analysis of variance followed by Bonferroni post hoc test: #*P*>.05. (**B**) Leiomyoma A006-8 F cells were pretreated with S1PR-specific antagonists (10 μmol/L W146, 1 μmol/L JTE013, or 5 μmol/L CAY10444) for 30 minutes before being challenged with 50 μg/mL activin A for 48 hours. Quantitative mRNA analysis of fibronectin and collagen 1A1 was performed with the use of real-time polymerase chain reaction in total RNA extracted from cells. Activin A increases the expression of fibronectin and collagen 1A1 in a statistically significant manner (Student *t* test: **P*<.05). The effect of $S1P_2$ or $S1P_3$ blockade by JTE013 or CAY10444 on activin A profibrotic effect is statistically significant according to two-way analysis of variance followed by Bonferroni post hoc test: #*P*>.05. S1P = sphingosine 1-phosphate; $S1P_{1–5}$ = S1P receptors 1–5; SK = sphingosine kinase.

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but was not affected by $S1P_1$ inhibition, suggesting a role for $S1P_2$ and $S1P_3$, but not $S1P_1$, in activin A–induced fibrosis.

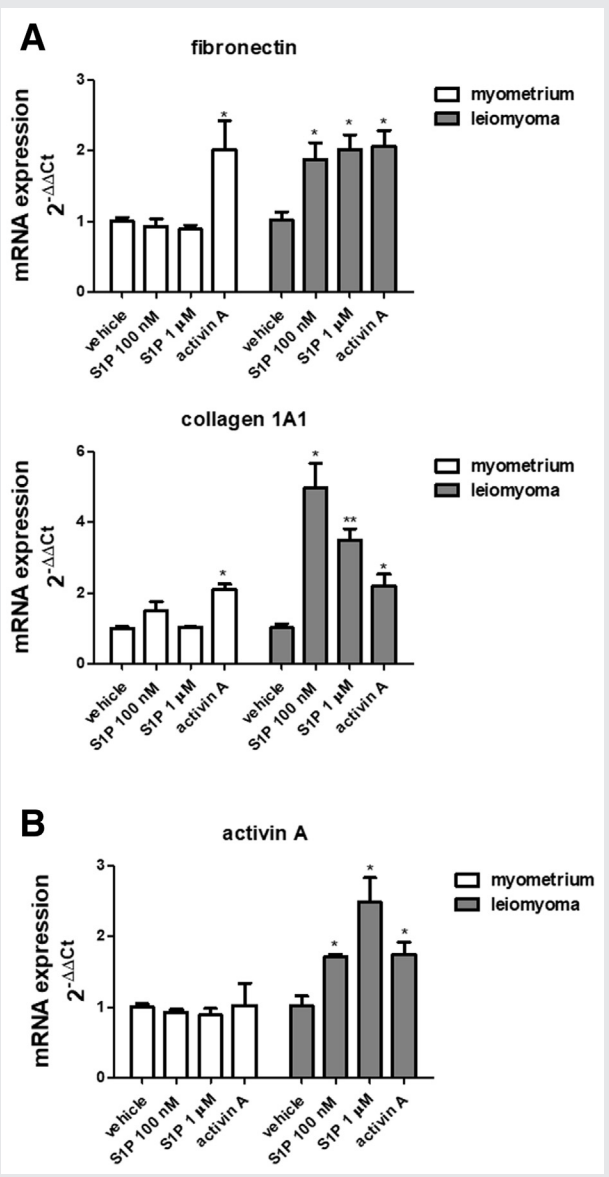
S1P Is a Profibrotic Cue in Leiomyoma Cells

To evaluate whether the bioactive lipid S1P directly promotes fibrosis in the onset and progression of leiomyoma, mRNA expression of fibronectin and collagen 1A1 was evaluated in A006-8 F cells treated with S1P (100 nmol/L or 1 μmol/L for 48 hours). Interestingly, S1P significantly up-regulated the mRNA expression of the fibrosis markers, demonstrating a profibrotic effect similar to that exerted by activin A (Fig. 4A). In contrast, in myometrial A005-7 cells S1P did not significantly alter the mRNA expression of the two fibrotic markers (Fig. 4A). Moreover, in leiomyoma A006-8 F cells, but not in myometrial A005-7 cells, S1P induced a remarkable increase of the expression of activin A (Fig. 4B). Activin A induced significant up-regulation of its own expression in leiomyoma but not in myometrial cells (Fig. 4B).

DISCUSSION

The present work shows for the first time a dysregulation of S1P signaling and metabolism in uterine fibroids: the expression of SK1 and SK2, the enzymes responsible for S1P biosynthesis, were significantly higher in leiomyoma than in adjacent myometrium. SK1 and SK2 both have a role in the induction of fibrosis in multiple organ systems (44). In murine models of kidney fibrosis, SK2 is up-regulated and SK2-deficient mice show less severe fibrosis (45), and gene deletion of *SK2* in bone marrow protects mice from folic acid–induced renal fibrosis (46). In skeletal muscle cells, the profibrotic action of TGF-β is mediated by SK1 (31). SK1, Spns2, and $S1P_2$ expression correlate with the severity of human liver fibrosis (47), and patients with idiopathic pulmonary fibrosis have

FIGURE 4



S1P profibrotic effect in leiomyoma cells. Quantitative mRNA analysis of fibronectin and collagen 1A1 was performed with the use of real-time polymerase chain reaction in total RNA extracted from A005-7 M cells and leiomyoma A006-8 F cells stimulated or not with S1P (100 nmol/L or 1 μmol/L) or 50 μg/mL activin A for 48 hours. Differences are statistically significant according to Student *t* test: **P*<.05; ***P*<.01. S1P = sphingosine 1-phosphate.

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increased S1P levels in serum and bronchoalveolar lavage (48).

Fibrosis is an exaggerated and continuous wound-healing process and is characterized by excessive production of ECM proteins. Uterine fibroids are considered fibrotic disorders because they contain 50% more ECM proteins than the corresponding myometrium (15, 49); therefore, considering the well recognized fibrotic phenotype of this disease, is crucial to understand the molecular mechanisms underlying the fibrotic process. The alteration of the ECM organization in uterine fibroids has been pointed out by means of X-ray phase-contrast imaging revealing higher mass collagen density distribution, higher collagen bundle thickness and fiber number, lower collagen fiber specific surface, and lower mean collagen bundles in leiomyoma tissues compared with myometrial tissues (50).

The present study also showed that $S1P_2$, $S1P_3$, and $S1P_5$ mRNA are significantly up-regulated in uterine fibroids compared with adjacent myometrium. We previously showed that $S1P_3$ is the most expressed receptor in skeletal muscle myoblasts after TGF-β1 treatment and that specific blockade of this receptor impaired the fibrogenic action of the cytokine (31). Augmented S1P levels in liver stimulate hepatic stellate cells toward fibrosis (47, 51), and inhibition of $S1P_2$ with a specific antagonist significantly diminished liver fibrosis in mice (24). Recently, a novel effect of $S1P_5$ on the inflammatory processes during low-dose bleomycin–induced fibrogenesis has been shown in murine skin (52). Accordingly, the antagonism of $S1P_2$, $S1P_3$, and $S1P_5$ might represent a potential therapeutic approach for uterine fibroids. Because S1PR redundancy may lead to limited efficacy, targeting multiple receptor isoforms may be more effective. Currently, a number of clinical trials are testing the effectiveness of S1PR-targeted drugs. FTY720, a sphingosine analogue that after in vivo phosphorylation acts on all S1PRs except $S1P_2$, is the first orally available agent approved by the Food and Drug Administration for the treatment of relapsing-remitting multiple sclerosis (53).

An original finding of the present study was that S1P signaling mediates the profibrotic action of activin A. In immortalized human leiomyoma cells but not in myometrial control cells, activin A increased the mRNA expression levels of SK1, SK2, and $S1P_2$. Interestingly, the profibrotic action of activin A was abolished when SK1/2 were inhibited and $S1P_{2/3}$ were blocked. The cytokine activin A is a member of the TGF-β superfamily isolated as an inducer of FSH secretion. It plays critical roles in cell proliferation, differentiation, apoptosis, and metabolism, as well as in mediating immunity, inflammation, wound repair, and fibrosis (54). Elevated expression of activin A increases the production of ECM in different pathologic conditions, including uterine fibroids (16). Activin A signaling through the Smad pathway has antiproliferative and/or fibrotic effects, depending on the cell type (myometrial or leiomyoma), being mainly antiproliferative in myometrial cells, while in leiomyoma cells the antiproliferative action is lost despite its fibrotic action (13). While extensive interplay has been previously shown between S1P and TGF-β (31, 55, 56), here, for the first time, a functional cross-talk between activin A and S1P signaling has been shown, unraveling previously unknown molecular mechanisms by which the cytokine evokes its fibrotic action in uterine fibroids.

Interestingly, S1P induced by itself the expression of fibrotic markers fibronectin and collagen 1A1 as well as of activin A selectively in leiomyoma but not in myometrial cells. Similarly, activin A stimulates its own transcription only in leiomyoma cells. These latter findings highlight the occurrence of a positive feedback toward the fibrotic phenotype exerted by S1P fueling activin A. In ELT3 rat uterine leiomyoma

cells, S1P has been reported to exert a proliferative and antiapoptotic action and SK1 mediates the antiapoptotic effect of endothelin-1 (57). Moreover, in the same cells, S1P is able of augmenting cyclooxygenase 2 expression via ABCC1 release and $S1P_2$ signaling (58).

CONCLUSION

The present findings show that the molecular mechanisms driving the fibrotic phenotype in uterine fibrosis crucially implicate a dysregulation of S1P signaling and metabolism. Overall, the functional cross-talk between activin A and S1P signaling open new perspectives for innovative pharmacologic targets to combat this fibrotic disease.

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Señalización de la Esfingosina 1-fosfato en miomas uterinos: implicación el efecto fibrótico de la Activina A.

Objetivo: Explorar el vínculo entre la señalización de esfingosina 1-fosfato (S1P) y el mioma y la posible intercomunicación de S1P con el efecto fibrótico de la Activina A.

Diseño: Estudio de laboratorio de caso-control

Lugar: Instituto Universitario y Hospital Universitario

Pacientes: Pacientes con miomas uterinos (n=26)

Intervención(es): Las muestras de tejido de mioma y miometrio normal fueron obtenidas de pacientes sometidas a miomectomía o histerectomía total.

Principales Medidas de Resultado: Se evaluaron los niveles de expresión de ARNm del enzima implicado en el metabolismo de S1P, receptores de S1P y el transportador de S1P Spns2 en muestras emparejadas de mioma/miometrio y población celular. Los efectos de la inhibición del metabolismo y señalización de S1P fueron evaluados sobre la acción fibrótica inducida por Activina A en las líneas celulares de mioma.

Resultado(s): La expresión de los enzimas responsables de la formación de S1P, esfingosina quinasa (SK) 1 y 2 y los receptores S1P2, S1P3 y S1P5 fueron significativamente aumentados en miomas comparado con adyacente miometrio. En las células de mioma, pero no en las células de miometrio, Activina A aumentó los niveles de expresión de ARNm de SK1, SK2 y S1P2. La acción profibrótica de la Activina A se abolió cuando Sk1/2 fueron inhibidos o S1P2/3 fueron bloqueados. Finalmente, S1P aumentó por sí mimo los niveles de ARNm de marcadores fibróticos (fibronectina, colágeno 1A1) y Activina A en miomas pero no en células miometriales.

Conclusión(es): Este estudio muestra que la señalización de S1P está desregulada en miomas uterinos y está involucrada en la fibrosis inducida por Activina A, abriendo nuevas perspectivas para el tratamiento de los miomas uterinos.

Palabras clave: Miomas uterinos, miomas, esfingosina 1-fosfato, receptores de esfingosina 1-fostato, esfingosina quinasa, fibrosis, Activina A.

RBMO

ELSEVIER

SHORT COMMUNICATION

Sphingosine 1-phosphate pathway is dysregulated in adenomyosis

BIOGRAPHY

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ABSTRACT

Research question: Is sphingosine 1-phosphate (S1P) pathway involved in the process of fibrosis in adenomyosis?

Design: RNA was extracted from paraffin-embedded slices collected from the ectopic endometrium of patients with nodular adenomyosis (n = 27) and eutopic endometrium of healthy controls women (n = 29). Expression of genes involved in the metabolism and signalling of S1P, and actin-alpha-2 smooth muscle, encoded by *ACTA2* gene, a gene involved in fibrogenesis, was evaluated by real-time polymerase chain reaction analysis.

Results: In adenomyotic samples, the expression of sphingosine kinase 1 (*SPHK1*), the enzyme responsible for the synthesis of S1P, and of S1P phosphatase 2 (*SGPP2*), the enzyme responsible for the conversion of S1P back to sphingosine, was lower (P = 0.0006; P = 0.0015), whereas that of calcium and integrin-binding protein 1, responsible for membrane translocation of SPHK1, was higher (P = 0.0001) compared with healthy controls. In S1P signalling, a higher expression of S1P receptor $S1P_3$ (P = 0.001), and a lower expression of $S1P_2$ (P = 0.0019) mRNA levels, were found compared with healthy endometrium. In adenomyotic nodules, a higher expression of *ACTA2* mRNA levels were observed (P = 0.0001), which correlated with $S1P_3$ levels (P = 0.0138).

Conclusion: Present data show a profound dysregulation of the S1P signalling axis in adenomyosis. This study also highlights that the bioactive sphingolipid might be involved in the fibrotic tract of the disease, correlated with the expression of *ACTA2,* suggesting its role as novel potential biomarker of adenomyosis.

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KEYWORDS

Adenomyosis
Alpha-smooth muscle actin
Ectopic endometrium
Fibrosis
Sphingosine 1-phosphate
Sphingosine 1-phosphate receptors

INTRODUCTION

Adenomyosis is a uterine disorder, defined by the presence of endometrial glandular and stromal tissue in the myometrium. It is associated with heavy menstrual bleeding, pelvic pain and infertility (*Vannuccini et al., 2017*).

Although the severity of the disease is known, its pathogenesis has not yet been completely elucidated. Fibrogenesis, characterized by progressive accumulation of extracellular matrix components, including collagen and alpha-smooth muscle actin, encoded by *ACTA2* gene, is a common finding in adenomyosis (*Vannuccini et al., 2017*).

Sphingosine 1-phosphate (S1P) is a bioactive lipid, which is a key molecule in fibrosis (*Donati et al., 2021*). Cellular levels of S1P are tightly regulated, depending on the relative rate between its biosynthesis, catalyzed by two different isoforms of sphingosine kinase (SPHK1 and SPHK2) and its degradation, occurring via two different pathways: the reversible dephosphorylation catalyzed by specific phosphatases (SGPP1 and SGPP2) and the irreversible cleavage by S1P lyase (SGPL). Most of S1P biological actions are evoked, after its extracellular release through transporters such as spinster homologue 2 (SPNS2), by its binding to specific G protein-coupled receptors named S1PR ($S1P_{1-5}$) (*Blaho and Hla, 2014*). The S1P signalling and metabolism are dysregulated in endometriosis where S1P axis was found to be implicated in mediating TGFβ fibrosis (*Bernacchioni et al., 2021*).

The present study aimed to investigate whether the expression of genes involved in S1P signalling axis is altered in adenomyosis.

MATERIALS AND METHODS

Patients and tissue collection

Tissue samples were collected from pathological formalin-fixed paraffin-embedded (FFPE) slices of non-pregnant and pre-menopausal woman who had undergone surgery (during the proliferative phase) at the Careggi University Hospital, Florence, Italy. In particular, ectopic endometrium of 27 patients aged 38–44 years affected by nodular adenomyosis and 29 control eutopic endometrial specimens of woman not affected by uterine disorders (age 30–42 years) were analysed.

All hormonal treatments were interrupted at least 3 months before surgery. The Institutional Review Board approved the study protocol (number 13742, 11 March 2019), and all patients gave informed written consent.

Quantitative real-time polymerase chain reaction

Total RNA extracted from FFPE tissue samples (500 ng) using the AllPrep DNA/RNA FFPE kit (Quiagen, Hilden, Germany) was reverse transcribed using the SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's recommendations. The quantification of target gene mRNAs was carried out in triplicate using TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Polymerase chain reaction cycling parameters were as follow: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min.

Target sequences were simultaneously amplified together with the reference gene β-actin (*ACTB*) (*Shen et al., 2019*; *Bernacchioni et al., 2021*; *Tian et al., 2022*). Relative quantification of mRNA expression was carried out through the $2^{-\Delta Ct}$ method (*Schmittgen and Livak, 2008*).

Statistical analysis

Student's t-test and Spearman correlation were was used for statistical analysis; $P < 0.05$ was considered to be statistically significant. Graphical representations were realized using GraphPad Prism 6.0 (GraphPad Software) (San Diego, CA, USA).

RESULTS

SPHK1 mRNA expression was significantly lower in adenomyosis than in control samples ($P = 0.0006$), whereas the expression of *SPHK2* was not significantly different (FIGURE 1A). Moreover, the mRNA levels of CIB1 (calcium and integrin-binding protein 1), a SPHK1-activating protein responsible for membrane translocation of the enzyme, was higher in adenomyosis than in control samples ($P = 0.0001$) (FIGURE 1A).

Catabolism of S1P showed lower levels of *SGPP2* mRNA in pathological samples compared with controls ($P = 0.0015$), whereas the expression of *SGPP1* and *SGPL* was not different between the two groups (FIGURE 1B).

The mRNA expression of $S1P_2$ resulted significantly lower in adenomyosis than in controls ($P = 0.0019$), whereas $S1P_3$ expression was significantly higher ($P = 0.001$) (FIGURE 1C). The other receptor isoforms and the transporter *SPNS2* showed no significant differences between the two groups (FIGURE 1C).

To correlate S1P signalling in adenomyosis with the fibrotic trait of the disease, the mRNA expression levels of fibrotic marker αSMA, encoded by *ACTA2* gene, were determined, which were significantly higher in adenomyosis than in controls ($P = 0.0001$) (FIGURE 1D). Interestingly, a Spearman correlation test between $S1P_3$ and *ACTA2* mRNA levels showed statistical significance ($P = 0.0138$), highlighting a possible role of S1P signalling in adenomyosis-associated fibrosis.

DISCUSSION

Further understanding of the molecular mechanisms that drive the onset and the progression of adenomyosis is urgently needed. To the best of our knowledge, the present study showed for the first time a dysregulation of mRNA expression of the genes involved in the metabolism and signalling of the bioactive sphingolipid S1P in adenomyosis, associated with an increased expression of the fibrotic marker αSMA.

The low levels of *SPHK1* mRNA observed in the study, with concomitant no changes of *SPHK2* levels, suggest a decrease in adenomyotic lesions of the SPHK1-synthetized S1P pool that can be exported out of the cells and bind to its specific receptors. The increased expression of the SPHK1-activating protein CIB1 in adenomyosis may probably compensate for the decreased amount of *SPHK1* transcript, making the enzyme more active. In addition, the reduced levels of the specific phosphatase *SGPP2* observed in adenomyosis may contribute to increase S1P levels in the lesions.

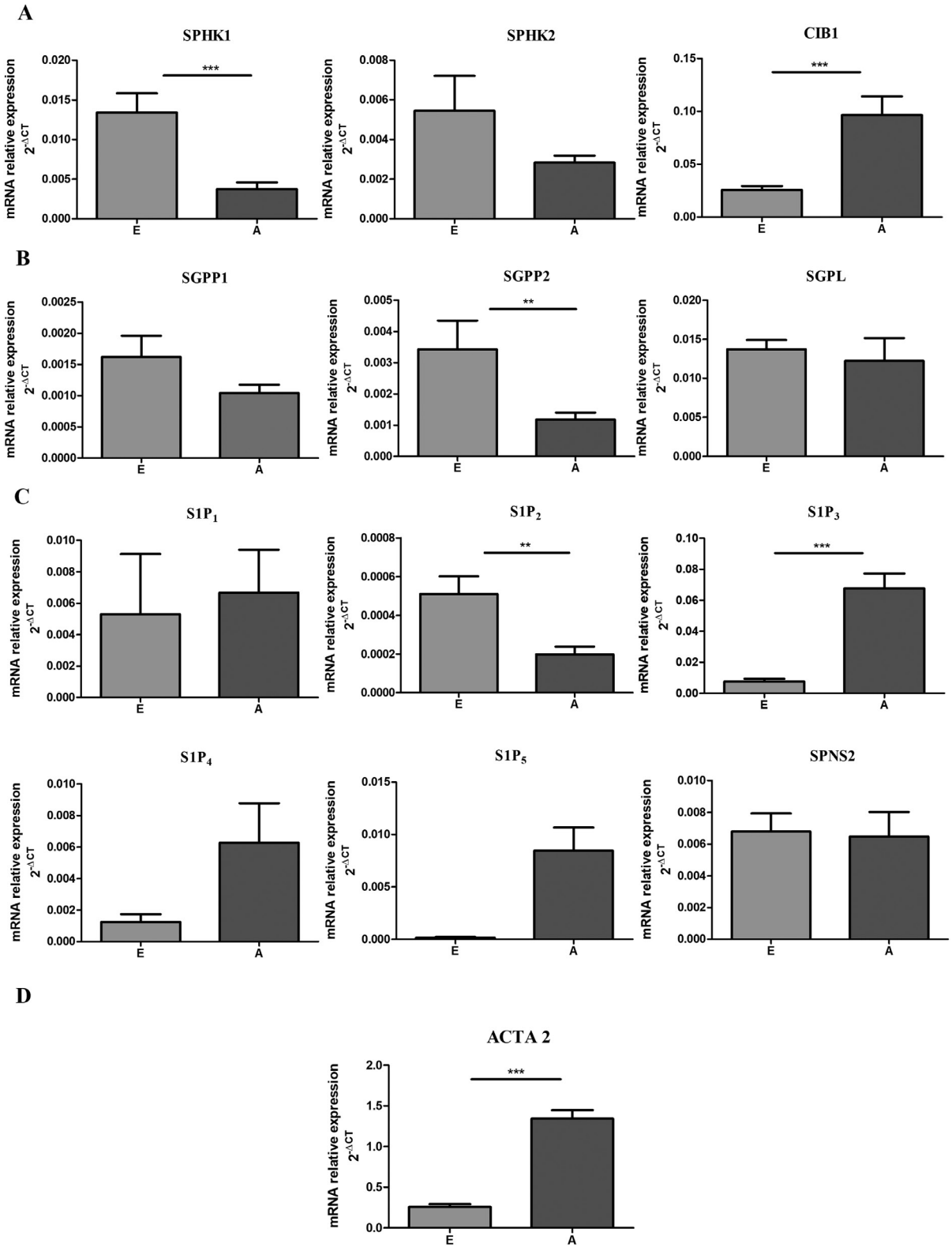

FIGURE 1 Sphingosine 1-phosphate (S1P) signalling axis is dysregulated in adenomyosis. Real-time polymerase chain reaction analysis was carried out in healthy eutopic endometrium (E) (n = 29), and ectopic endometrium of woman affected by adenomyosis (A) (n = 27) using TaqMan Gene Expression Assay probes specific for sphingosine kinases (SPHK1, SPHK2) and SPHK1-modulating protein CIB1 (A), S1P phosphatases (SGPP1, SGPP2) and S1P lyase (SGPL) (B), S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$) and S1P transporter spinster homologue 2 (SPNS2) (C) and the fibrotic marker αSMA, encoded by *ACTA2* gene (D). Results were analysed with the use of the $2^{-\Delta Ct}$ method. Differences are statistically according to Student's t-test (**P < 0.01, ***P < 0.001). The exact P-values were: SPHK1, P = 0.0006; CIB1, P = 0.0001; SGPP2, P = 0.0015; $S1P_2$, P = 0.0019; $S1P_3$, P = 0.001; ACTA2, P = 0.0001.

The decreased expression shown in adenomyotic nodules of $S1P_2$, which is reported to inhibit cell migration (*Blaho and Hla, 2014*), suggest a possible involvement of S1P signalling in the increased migration of adenomyotic cells. Moreover, *S1P*$_3$ mRNA levels were found to be significantly higher in adenomyosis than in control samples: this receptor isoform is involved in the onset of fibrosis in different tissues (*Blaho and Hla, 2014*; *Donati et al., 2021*).

According to the relevance of fibrosis in adenomyosis, the present study highlights increased expression levels of the fibrotic marker αSMA, encoded by *ACTA2* gene, that statistically correlated with *S1P*$_3$ mRNA levels, highlighting a possible implication of S1P signalling via $S1P_3$ in the fibrotic trait associated with adenomyosis. These data suggest that the pharmacological blockade of $S1P_3$ by fingolimod (FTY720), the first orally available agent FDA approved for the treatment of relapsing-remitting multiple sclerosis and efficacious in counteracting fibrosis in different tissues (*Donati et al., 2021*), may be studied in adenomyosis.

In conclusion, the present study shows that an altered S1P signalling pathway may be involved in the fibrotic phenotype of adenomyotic nodules identifying a possible new target for counteracting this uterine disorder.

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