The role of the sympathetic nervous system and semaphorins in the pathogenesis of endometriosis

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Contents

Abbreviation Index

ADR	adrenaline
CNS	central nervous system
DOP	dopamine
E2	estrogen
EM	endometriosis
EtOH	ethanol
h	hour
HP	healthy peritoneum
IF	Immunofluorescence straining
IHC	Immunohistochemical staining
IL	interleukin
ISH	in situ hybridization
MMP	matrix-metalloproteinases
NAD	noradrenaline
ND	no data
NGF	neural growth factor
Nrp 1	neuropilin 1
Nrp 2	neuroilin 2
OC	oral contraceptiva
PCR	polymerase chain reaction
Plxn	plexin
PNS	peripheral nervous system
Sema 3C	semaphorin 3C
Sema 3F	semaphorin 3F
TGF	transforming growth factor
TH	tyrosin hydroxylase
TNF	Tumor necrosis factor
UP	unaffected peritoneum
VEGF	vascular endothelial growth factor

1 Introduction

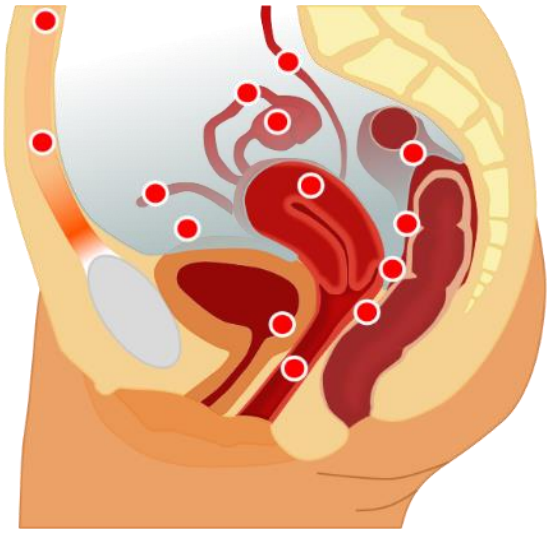
1.1 Definition of endometriosis

Endometriosis is a chronic inflammatory gynecological disease, characterized by the appearance of ectopic endometrium-like cells (glands and stromal cells). This estrogen dependent disease is a common condition, which affects around 15% of all women in reproductive age. The course of the disease is benign, though mostly accompanied by chronic pelvic pain and infertility.

The endometriosis lesions appear as endometriosis implants on the surface of the pelvis, peritoneum, or pelvic organs. Also possible is the invasion of ovaries developing ovary cysts, or infiltration in adjacent organs (deep infiltrating endometriosis) like the intestine, or into tissue between vagina and rectum forming nodules (rectovaginal endometriosis). The endometriosis lesions stimulate the development of fibrotic tissue, due to their inflammatory and irritating effects, leading to the emergence of adhesions covering pelvic organs or binding them together. This might be responsible for a dysfunctional ovulation procedure.

Endometriosis is classified in different forms:

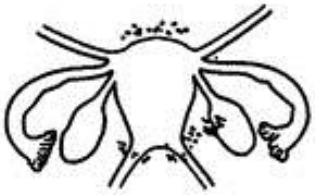
- Endometriosis genitalis externa: lesions in the pelvis, peritoneum, uterine ligaments, ovaries and douglas cul-de-sac.
- Endometriosis genitalis interna: lesions in the uterus and fallopian tubes musculature (myometrium), also called adenomyosis and fallopian endometriosis.
- Endometriosis extragenitalis: lesions in adjacent organs, for example gut or bladder.



Derivative work: Hic et nunc – female anatomy (by Tsaitgaist)

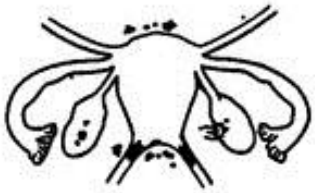
Depending on different aspects as location, extent, depth of the endometriosis implants; presence and severity of adhesion; and presence and size of ovarian cysts, the endometriosis genitalis externa is classified in four stages: I-minimal, II-mild, III-moderate, and IV severe (Medicine, 1997). Endometriosis stage and presence or severity of symptoms show no correlation, though infertility is common with stage IV endometriosis, probably due to severe adhesions.

STAGE I (MINIMAL)



PERITONEUM		
Superficial endo	– 1–3cm	–2
R OVARY		
Superficial endo	– < 1cm	–1
Filmy adhesions	– < 1/3	–1
TOTAL POINTS		4

STAGE II (MILD)

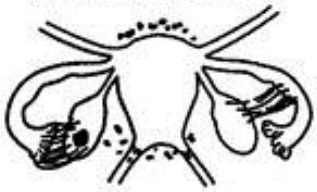


PERITONEUM		
Deep endo	– > 3cm	–6
R OVARY		
Superficial endo	– < 1cm	–1
Filmy adhesions	– < 1/3	–1
L OVARY		
Superficial endo	– < 1cm	–1
TOTAL POINTS		9

STAGE III (MODERATE)

PERITONEUM		
Deep endo	– > 3cm	–6
CULDESAC		
Partial obliteration		–4
L OVARY		
Deep endo	– 1–3cm	–16
TOTAL POINTS		26

STAGE III (MODERATE)



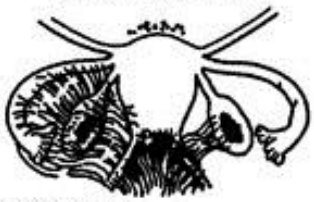
PERITONEUM		
Superficial endo	– >3cm	–4
R TUBE		
Filmy adhesions	– < 1/3	–1
R OVARY		
Filmy adhesions	– < 1/3	–1
L TUBE		
Dense adhesions	– < 1/3	–16*
L OVARY		
Deep endo	– < 1cm	–4
Dense adhesions	– < 1/3	–4
TOTAL POINTS		30

STAGE IV (SEVERE)

PERITONEUM		
superficial endo	– > 3cm	–4
L OVARY		
Deep endo	– 1–3cm	–32**
Dense adhesions	– < 1/3	–8**
L TUBE		
Dense adhesions	– < 1/3	–8**
TOTAL POINTS		52

*Point assignment changed to 16
**Point assignment doubled

STAGE IV (SEVERE)



PERITONEUM		
Deep endo	– >3cm	–6
CULDESAC		
Complete obliteration		–40
R OVARY		
Deep endo	– 1–3cm	–16
Dense adhesions	– < 1/3	–4
L TUBE		
Dense adhesions	– > 2/3	–16
L OVARY		
Deep endo	–1–3cm	–16
Dense adhesions	–> 2/3	–16
TOTAL POINTS		114

(ASRM, 1997) (Medicine, 1997)

1.2 Symptoms and diagnosis of endometriosis

Commonly, endometriosis progression is chronical and painful. More than half of the affected women suffer of dysmenorrhea and two out of three experience painful intercourse (dyspareunia). Heavy bleeding is a further common symptom, unlike dysuria and dyschezia, the incidence is rather lower, often dependent of the involvement of organs as gut and bladder. However, during peritoneal endometriosis, especially chronical pelvic pain persists (Kaiser, et al., 2009).

Infertility is a further feature endometriosis implicates, up to 50% of infertile women have endometriosis. Despite the strong association between endometriosis and infertility, not all endometriosis patients are infertile. Some cases of tubal sterilization or other laparoscopic procedures without suspected endometriosis, show incidental finding of endometriosis. This are in the most cases women without severe or absolutely symptoms (Medicine, 1997).

First diagnosis occurs by analysis of the symptoms given above. Gynecological examination can reveal nodules, an inclined position of the uterus or even a retroverted uterus. Further, fixation or enlargement of the ovaries can be determined. In few cases, it is possible to find the endometriosis lesions in vagina or cervix (macroscopic).

The differential diagnosis complicates the determination of endometriosis affection. Many similar symptoms occur in several other chronic-inflammatory diseases such as inflammatory bowel disease or irritable colon (Schindler, 2008). Therefore, the determination of the symptoms is not sufficient to determine the medical condition of endometriosis and confirmation of the final diagnosis has to take place by laparoscopy.

The difficult conditions for diagnosing endometriosis cause a prolongation of time until final diagnosis takes place, about 6-8 years after appearance of the first symptoms (Schweppe, 2003).

1.3 Etiology of endometriosis

The root of the emergence of endometriosis remains uncertain. Nevertheless, the discovery of endometriosis was in 1860 (Batt, 2011) and since then many theories have been developed.

The *implantation theory* by John A. Sampson (Sampson, 1927) is the most widely accepted theory. Retrograde menstruation is a known process during the menstruation of women; hereby menstrual blood containing endometrial cells flows back through the fallopian tubes reaching the peritoneal cavity. The implantation theory suggests an implantation of these endometrial cells on the peritoneum and surfaces of organs in the peritoneal cavity, establishing endometriosis lesions. Nevertheless, retrograde menstruation is a known phenomenon occurring in most women. During endometriosis, the immune system might not be able to degrade properly the cell debris in the peritoneal cavity, which would suggest an impairment of it in endometriosis patients. An increase of macrophages and macrophage-derived secretion products, is characteristic during endometriosis, nevertheless it remains unclear if the increase is contributing to the maintenance of the condition or if it is simply a product of the immune reaction. By now, it is still not clear if there is a relationship between autoimmune disease and endometriosis. Not only the immune system, but also other factors such as toxins or hereditary factors might contribute to the implantation. Normally, after retrograde menstruation, the immune system is able to get rid of the cell debris in the peritoneal cavity and thereby prevents implantation and growth of these cells at ectopic places, which is not the case in endometriosis patients. Obviously, in women with endometriosis additional unknown factors provoke or at least support the implantation and growth of the endometrial cells in the peritoneal cavity. Therefore, retrograde menstruation alone is not an explanation for the emergence of endometriosis.

The implantation theory approach is extended by the *tissue injury and repair concept* by Leyendecker and explains why most women have retrograde menstruation but are not always affected with endometriosis. Here, the uterus is supposed to promote the detachment and dissemination of stem cells from the basalis of the endometrium and triggers the differentiation of these cells into endometrial lesions. It is suggested that this process is initiated due to a hyperperistalsis of the uterus. Possibly, through the tissue repair mechanisms a rise of estrogen occurs, which could be responsible for the maintenance of the hyperperistalsis and lead to auto-traumatization (Leyendecker, et al., 1998).

The *coleomic metaplasia theory* suggests metaplastic degeneration of embryonic cells triggered by specific stimuli, leading to endometriosis. Inflammatory or hormonal

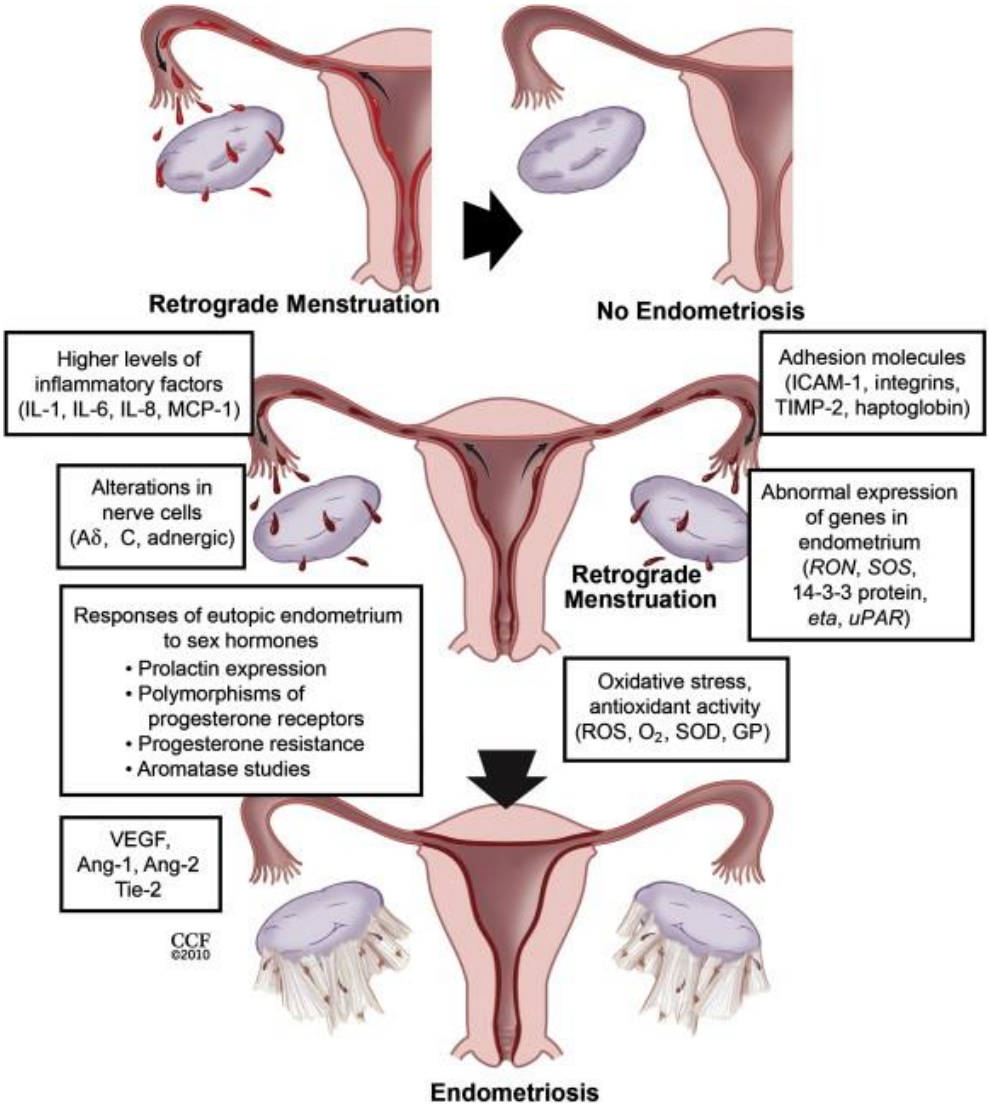
factors could be responsible for the initiation of the metaplasia (Meyer, 1919). This theory is able to explain the appearance of endometriosis outside the pelvic cavity; in girls before the menarche; and in men that received an estrogen treatment due to a prostectomy. Nevertheless, there are many contradictory factors known against this theory (Oliker and Harris, 1971, Pinkert, et al., 1979, Schrodt, et al., 1980).

However, until now none theory is able to explain fully the emergence and development of the disease endometriosis. The analysis lead to results of which is hard to distinguish if it is a matter of cause or if it is an effect of the disease. Because the principle of endometriosis is not well known, it is difficult to elaborate a proper treatment for the affected ones.

1.4 Molecular mechanisms in the pathogenesis of endometriosis

The pathogenesis of endometriosis is based on migration, adhesion, invasion, proliferation and angiogenesis. Predisposing factors such as inflammation, metabolic changes, formation of ectopic endometrium and generation of pain and other effects might play an important role. Nevertheless, the pathophysiology of endometriosis is likely to be multifactorial and to involve an interplay between several factors. Factors expressed in eutopic and ectopic endometrium of women with endometriosis but not in the eutopic endometrium of women without endometriosis, are suggested to be responsible for the migration.

Leyendecker et. al suggested that the basalis, which shows certain properties of stem cells, has a greater potential to lead to dislocation and proliferation in women with endometriosis than in healthy women (Leyendecker, et al., 2002). For the adhesion of endometrial cells to the peritoneum, the mesothelial cells need to be exposed. Exposition of the mesothelial cells occurs after tissue injury or because of inflammatory processes, which might be the case during endometriosis (Demir Weusten, et al., 2000, Koks, et al., 1999).



(Carvalho, et al., 2011)

The invasion is probably mainly regulated by cadherins and matrix-metalloproteinases (MMP). Cadherins are cell-cell contact proteins, which contribute to preserve the structure of the endometrium. Therefore, the loss of E-Cadherin during endometriosis is presumably crucial for invasion (Starzinski-Powitz, et al., 1999). MMPs are expressed in eutopic endometrium and are cyclic regulated. In the endometriosis context, MMPs cause a degradation of the extra cellular matrix, thereby promoting invasion. MMPs expression can be induced by hormones and inflammatory cytokines, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor necrosis factor alpha (TNF-α) (Nap, 2012), which might point at an induction of MMPs during endometriosis, due to the inflammatory milieu.

Interestingly, endometriosis reveals several properties of an autoimmune disease, e.g. increased B-cell activity or abnormalities in T- and B-cell function (Lebovic, et al., 2001, Nothnick, 2001). Additionally, women with endometriosis are oftener affected by autoimmune diseases and genome-wide transcriptional profiling analysis revealed gene expression patterns similar to other autoimmune disorders, supporting the autoimmune facet of endometriosis (Hever, et al., 2007, Sinaii, et al., 2002).

Interleukin-4 (IL-4) is a well known factor during autoimmunity and might therefore play an important role during endometriosis. During inflammation and wound repair, IL-4 induces M2 macrophage activation, which is leads to IL-10 and transforming growth factor-β (TGF-β) secretion and therefore results in decrease of inflammation (Kiguchi, et al., 2015, Lech and Anders, 2013). In endometriosis, IL-4 concentration in serum and peritoneal fluid of adolescent girls suffering from chronic pelvic pain is significantly higher than in serum and peritoneal fluid of the control without endometriosis (Drosdzol-Cop, et al., 2012). Furthermore, expression of IL-4 in endometriotic stromal cells was demonstrated and suggested to play a crucial role during the proliferation of endometrial and stromal cells (Giudice, et al., 2012, Park, et al., 2009).

TGF-β is a multifunctional cytokine, which plays a crucial role in wound healing, tissue repair and fibrosis. In injured tissue, TGF-β leads to an increase of matrix protein synthesis and simultaneously a decrease of matrix protein degradation, thereby resulting in tissue fibrosis (Branton and Kopp, 1999). During wound healing, TGF-β is suggested to promote the ability of fibroblasts to contract collagen ant thereby secure a provisional wound matrix (Montesano and Orci, 1988). Additionally, TGF-β is involved in fibroblast recruitment and activation (Kalluri and Zeisberg, 2006, Ronnov-Jessen and Petersen, 1993).

Expression of TGF-β has been demonstrated in endometrium, where endometrial cells and macrophages are responsible for its secretion (Jones, et al., 2006, Wu and Ho, 2003). In endometriosis, TGF-β is suggested to play a major role during the establishment and maintenance of endometriosis, since it is potentially secreted into the peritoneal fluid of women with endometriosis (Omwandho, et al., 2010).

Importantly, fibroblasts activation is crucial during inflammation. Generally, fibroblasts are known as producers of extracellular matrix and as the most abundant cell type in stroma (Chang, et al., 2002). Nevertheless, fibroblasts can produce and respond to

several growth factors, thereby contributing to the maintenance of the homeostasis of epithelial and endothelial cells. Consequently, fibroblasts are key regulators during tissue development, differentiation and repair (Kalluri and Zeisberg, 2006). During immune response, fibroblasts produce cytokines and chemokines, and are therefore crucial for the development and modulation of the immune response (Anderson and Jenkinson, 2001). Additionally, modification of the quality, quantity and duration of inflammatory infiltrates during the emerging of inflammatory responses, has been attributed to fibroblasts (Parsonage, et al., 2005). Consequently, fibroblasts normalize chemokine gradients, thereby leading to apoptosis of the infiltrating leukocytes; altogether contributing to the resolution of the inflammation (Buckley, et al., 2001). Therefore, a review study from 2008, proposed fibroblasts as novel anti-inflammatory therapeutic targets in chronic inflammation (Flavell, et al., 2008).

Angiogenesis is a further crucial factor during the pathogenesis of endometriosis. For the survival of every organ or tissue, blood supply is necessary. In the same manner, endometriosis implants require sufficient blood supply to obtain oxygen and nutrients essential for survival and development (Groothuis, et al., 2005). In this context, vascular endothelial growth factor (VEGF) seems to play an important role. Several studies demonstrated an increase of VEGF levels in endometriotic tissue and consequently an increase of vascularization in the peritoneum, thereby promoting the implantation and survival of endometrial cells in the retroperitoneal space (Donnez, et al., 1998, McLaren, et al., 1996).

Furthermore, endometriosis is known to cause severe pelvic pain. Even though the mechanisms leading to pain are sparsely known, it is suggested that the chronic inflammatory state in endometriosis, which leads to high production rates of interleukins, histamines and different growth factors might result in pain. Specifically IL-1, IL-2, IL-6, IL-10, TNF-α, interferon (IFN)-γ, have been implicated in the pain pathogenesis of endometriosis. Increased levels of IL-10 have been demonstrated in peritoneal fluid and medium from cultured peritoneal macrophages of women with endometriosis (Ho, et al., 1997, Rana, et al., 1996, Wu, et al., 1999). IL-10 can regulate T helper cells to direct Th1 or Th2 dominance, which act pro-inflammatory and anti-inflammatory respectively (Kennedy, et al., 1994).

In addition, the endometriotic innervation could be highly involved in pain emergence during endometriosis (Atwal, et al., 2005, Stratton and Berkley, 2011, Wang, et al.,

2009). As mentioned above, endometriosis lesions are highly vascularized and vessels are often accompanied by nerve fibers, which can act either pro- or anti-inflammatory and thereby contribute to the inflammatory status and further to pain emergence. Nevertheless, the molecular mechanisms regulating endometriotic innervation remain widely unknown.

Ultimately, the exact mechanisms leading to disease and/or pain emergence remain unclear.

1.5 Angiogenesis in endometriosis

During normal reproduction, cyclic angiogenesis provides the necessary signals for follicular maturation, corpus luteum function, endometrial growth and remodeling (Jaffe, 2000). In endometriosis, angiogenesis plays an important role. For instance, the success of ectopic implants depends on pathological processes such as angiogenesis. A review study from 2013, suggested a model where the endometrial cells, which reach the peritoneal cavity induce an inflammatory response, thereby inducing adhesion, angiogenesis and peritoneal invasion. Further leading to endometriotic implants, which secrete proangiogenic factors (e.g. VEGF, IL-1β, IL-8, TNF-α, IL-6, etc.), which then induce neovascularization and the survival of the endometriotic implants in the peritoneal cavity (Rocha, et al., 2013).

Interleukin 1β (IL-1β), is the dominant IL-1 secreted by activated peritoneal macrophages in response to refluxed endometrium. Since IL-1β can induce VEGF and IL-6 production, and it was shown to be able to activate an angiogenic phenotype in stromal cells of endometriosis lesions but not in normal endometrium cells, it is suggested to play an important role in the neovascularization during endometriosis.

IL-6 is known as a protein promoting endometrial cell proliferation and angiogenesis (Cohen, et al., 1996, Giudice, 1994). It has been shown that IL-6 secretion is increased in ectopic endometrial tissue and peritoneal fluid of women with endometriosis(Keenan, et al., 1994).

VEGF is a renowned vascular permeability factor and crucial stimulator of physiological and pathological angiogenesis (Ferrara, 2009, Nagy, et al., 2007). It also contributes

to endothelial cell proliferation, migration, organization into tubules, altogether participating in the angiogenic cascade (Mueller, et al., 2000).

The expression of VEGF seems to be cycle dependent, having the maximal expression during the secretory phase and menstruation (Shifren, et al., 1996). During late proliferative phase, the neovascularization and increased vascular permeability correlate with endometrial VEGF expression, which is enhanced by estradiol (Charnock-Jones, et al., 2000). The production and secretion of VEGF can be attributed to activated peritoneal macrophages and further to neutrophils (McLaren, et al., 1996, Mueller, et al., 2000). In endometriosis, VEGF expression can be found in endometriotic lesions and elevated levels are found in the peritoneal fluid of endometriosis patients (Donnez, Smoes, Gillerot, Casanas-Roux and Nisolle, 1998, Shifren, Tseng, Zaloudek, Ryan, Meng, Ferrara, Jaffe and Taylor, 1996).

VEGF acts through binding to membrane bound receptors, which transduce its signal. One of the main receptors is Neuropilin 1 (Nrp1), which is known as a class 3 semaphorin receptor. In addition, Nrp1 is known as a key factor during angiogenesis. The phenotype of mutant mice with Nrp1 gene deletion has abundant vascularization, dilated blood vessels and wide hemorrhage, similar to mutant mice with excessive VEGF expression, suggesting possible modulation of angiogenesis through a VEGF-Nrp1 signaling pathway (Becker, et al., 2005, Gu, et al., 2003, Kitsukawa, et al., 1995).

Angiogenesis is a crucial factor in the establishment and pathogenesis of endometriosis; therefore, it is a potential new target for therapy. Several studies in endometriosis models have shown a regression of endometriotic lesions after blood supply reduction (Nap, 2004, Ozer, et al., 2013, Ricci, et al., 2011). Nevertheless, before anti-angiogenic agents can be applied in clinical practice, several studies have to be performed to elucidate possible consequences or unwanted side effects.

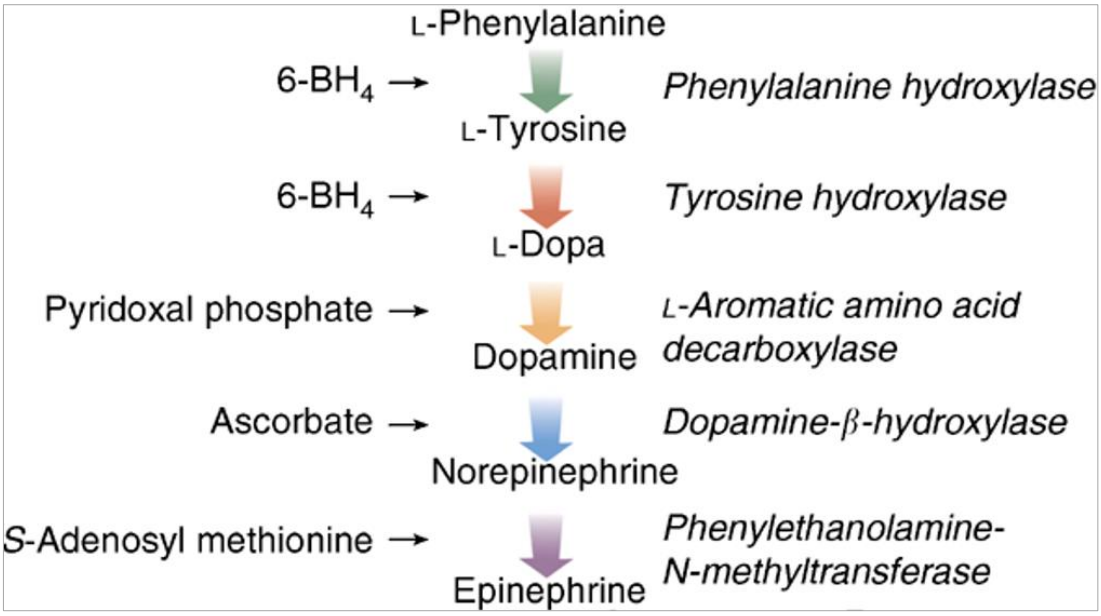
1.6 The nervous system and immune response in endometriosis

The nervous system in humans is responsible of coordinating voluntary and involuntary actions by transmitting signals to and from different parts of the body. It is divided in two main parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of brain and spinal cord. The PNS consists of nerves, which connect the CNS to the rest of the body. Furthermore, the PNS is divided into the

somatic, autonomic and enteric nervous system. The sympathetic and parasympathetic nervous systems are part of the autonomic nervous system. The sympathetic nervous system controls the body's fight or flight response and maintains homeostasis, while the parasympathetic nervous system stimulates the body to rest and digest.

Sympathetic nerves arise from near the middle of the spinal cord. The axons leave the spinal cord and pass near the spinal sensory ganglion to enter the anterior rami of the spinal nerves. Consequently, they connect the paravertebral or prevertebral ganglia to extend alongside the spinal column, giving rise to the sympathetic chain ganglia.

The sympathetic neurons release acetylcholine, leading to a great secretion of adrenaline and to a lesser extent of noradrenaline (also known as norepinephrine), which activates adrenergic receptors. Postganglionic sympathetic neurons release catecholamines, i.e. adrenaline (also known as epinephrine) and to a higher extent noradrenaline (McCorry, 2007). For the synthesis of adrenaline, the amino acid tyrosine is first oxidized to L-DOPA; consequently, L-DOPA is decarboxylated resulting in dopamine, which is oxidized into noradrenaline. Methylation of the primary amine of noradrenaline gives rise to adrenaline (Siegel and Agranoff, 1999). Thereby, tyrosine hydroxylase is responsible for the conversion of L-tyrosine to L-DOPA; hence, tyrosine hydroxylase is expressed in sympathetic nerves and is often used as a specific marker for noradrenergic innervation (Elenkov, et al., 2000).



(Grando, et al., 2006)

There is increasing evidence that malfunctions in the sympathetic neurotransmitter expression is profoundly involved in the mechanisms of chronic inflammation (Koopman, et al., Straub, Straub and Harle).

Until now, the exact mechanisms leading to disease- and/or pain-emergence in endometriosis remain unclear. Nevertheless, parts of the sensory and sympathetic nervous system could play an important role.

1.7 Innervation and immune response in endometriosis

The decrease of sympathetic nerve fibers seems to be a pathologic feature common in several chronic inflammatory diseases, like rheumatoid arthritis, psoriasis, Crohn´s disease, pruritus, peritoneal endometriosis or adenomyosis (Miller, et al., 2000) (Weidler, et al., 2005) (Haas, et al., 2010) (Arnold, et al., 2012, Barcena de Arellano, et al., 2013, Lehner, et al., 2008). However, the mechanisms leading to the decrease remain misunderstood. The sympathetic nervous system is involved in the development and severity of chronic inflammatory diseases (Capellino and Straub, 2008, Straub, 2007). Furthermore, the sympathetic hypoinnervation in the inflamed tissue is accompanied by a sensory hyperinnervation, consequently leading to an imbalance between nerve fibers, which respectively release inflammatory or anti-

inflammatory neurotransmitters (Arnold, Barcena de Arellano, Ruster, Vercellino, Chiantera, Schneider and Mechsner, 2012, Miller, Justen, Scholmerich and Straub, 2000, Weidler, Holzer, Harbuz, Hofbauer, Angele, Scholmerich and Straub, 2005). An imbalance in neurotransmitter signaling promotes the maintenance of a chronic inflammatory stage (Miller, Justen, Scholmerich and Straub, 2000, Straub and Harle, 2005).

Importantly, the nervous system and the immune system interact and work with each other to modulate the immune response (Straub, 2007). In fact, the modulation of the sensory and sympathetic nervous system is highly influenced by immune cells such as macrophages, mast cells, B- and T-cells (Kingery, 2010, Koopman, Stoof, Straub, Van Maanen, Vervoordeldonk and Tak, 2011, Lakhan and Kirchgessner, 2010, Pongratz and Straub, 2010). Whereas, immune cells and thereby the immune system is tuned by noradrenaline, which is released locally, or adrenaline, which is secreted by the adrenal medulla (Elenkov, Wilder, Chrousos and Vizi, 2000, Nance and Sanders, 2007). Noradrenaline and adrenaline are catecholamines, which were generally known as immunosuppressive. Nevertheless, it has been shown that catecholamines can also have immunepromotive effects, which explains contradictory effects of the neuroendocrine system on the immune response during infections, autoimmune diseases or inflammation (Elenkov, Wilder, Chrousos and Vizi, 2000, Miller, Justen, Scholmerich and Straub, 2000).

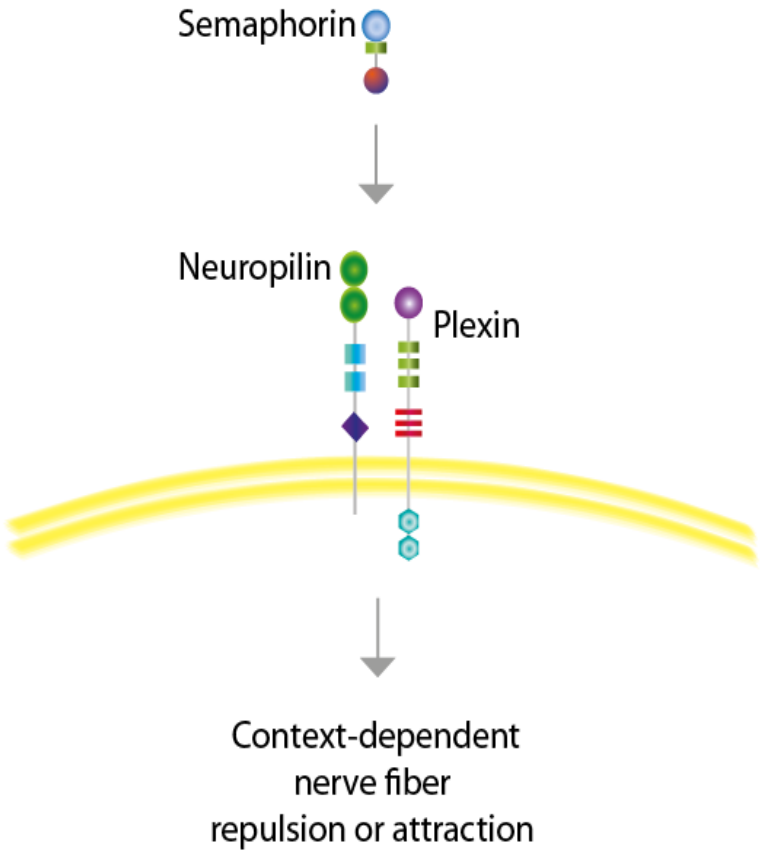
Furthermore, during chronic inflammation, the infiltration of macrophages causes the formation of fibrosis, thereby supporting the inflammatory process (Ricardo, et al., 2008). In endometriosis, increased levels of macrophages in peritoneal fluid and peritoneum were shown by several researchers (Montagna, et al., 2008, Tran, et al., 2009). It is suggested that the peritoneal fluid of patients with endometriosis have neuromodulatory effects and might play a role during the modulation of sensory and sympathetic outgrowth during the disease (Barcena de Arellano, et al., 2011). Nevertheless, the exact mechanisms regulating these actions are still to be elucidated.

Studies in rheumatoid arthritis as wells as in Crohn´s disease proposed specific nerve repellent factors (semaphorins) as potential effectors in the depletion of sympathetic nerve fibers in the inflamed tissue (Miller, et al., 2004, Straub, et al., 2008).

1.8 Semaphorins - types and signal transduction

Semaphorins (Semas) are highly conserved membrane bound or secreted proteins, originally known from neural development as axonal guidance molecules. Semas transduce short-range signals, usually acting as nerve repellent factors deflecting axons from inappropriate regions. The transduction of Semas signaling occurs through binding multimeric receptor complexes (neropilins and plexins).

Semas are classified into eight major classes. The first seven classes are class 1 to class 7. The eighth class is class V. Classes 1 and 2 are only found in invertebrates; class 3, 4, 6, and 7 are only found in vertebrates. Class 5 can be found in both, and class V is specific to viruses. Furthermore, each class of Sema is additionally sub grouped into different molecules, which share similar characteristics.



1.9 Semaphorins in endometriosis

Class 3 Semas are secreted proteins having their function during neural development, nevertheless recent studies have elucidates potential functions of these semaphorins in the nervous system after development is finished (Kikutani, et al., 2007, Sharma, et al., 2012). As the other semaphorin classes, Semas class 3 transduce their signal through binding to heterocomplexes consisting of neuropilins (Nrp) and class A plexins (Plxn) (Chen, et al., 1997, Giger, et al., 1998).

The affinity of the receptors with specific semaphorins varies between the different Semas and the signal, which is supposed to be transduced. Sema3C requires binding to Nrp1 and Nrp2 to induce sympathetic nerve fiber collapse (Chedotal, et al., 1998, Giger, Pasterkamp, Holtmaat and Verhaagen, 1998, Messersmith, et al., 1995, Takahashi, et al., 1998). Other than Sema3C, Sema3F can bind Nrp1, but rather binds Nrp2, since the affinity to Nrp2 is 10-fold greater.(Chen, Chedotal, He, Goodman and Tessier-Lavigne, 1997). However, Nrp's are membrane bound receptors with an undersized intracellular domain, which impedes their possibility to transduce a signal. Therefore, Nrp's are compelled to build complexes with further receptors, which do have the capability of transducing the signal, and these are represented by the plexins. Especially PlxnA3 and PlxnA4 have been demonstrated in the receptor complex favored by Sema3F, even though the affinity of Sema3F is higher to PlxnA3 than to PlxnA4 (Cheng, et al., 2001, Waimey, et al., 2008, Yaron, et al., 2005).

Overexpression of Sema3C and Sema3F was demonstrated to be in high correlation with reduced appearance of sympathetic nerve fibers in several diseases (Fassold, et al., 2009, Graf, et al., 2012). Furthermore, the expression of semaphorins was attributed to macrophages and fibroblasts present during tissue injury and inflammation and where suggested to contribute to the chronification of the inflammation (Ji, et al., 2009, Miller, Weidler, Falk, Angele, Schaumburger, Scholmerich and Straub, 2004, Pasterkamp, et al., 1999).

As well known, endometriosis is an estrogen dependent disease. Interestingly, elevation of estrogen levels lead to an increase of Sema3F expression and additionally leads to the depletion of sympathetic nerve fibers in an in vivo model (Klatt, et al., 2012, Richeri, et al., 2011). In vivo studies of an arthritis mouse model, revealed that Nrp2, the main Sema3C and 3F receptor, aggravates sympathetic nerve repulsion, and even aggravates the arthritis score. Furthermore, Nrp2 can be located on the surface of

sympathetic nerve fibers and is then responsible for their repulsion when induced by Sema3C or 3F (Chen, Chedotal, He, Goodman and Tessier-Lavigne, 1997, Fassold, Falk, Anders, Hirsch, Mirsky and Straub, 2009, Ruediger, et al., 2013). The expression of Nrp2 on sympathetic nerve fibers is usually limited to the time during neural development, therefore the appearance of Nrp2 expression after neural developmental events have finished, is based on a tissue injury, a pathological event or neuronal regeneration (De Winter, et al., 2002).

Therefore, sympathetic nerve repulsion in chronic inflammatory diseases (such as rheumatoid arthritis) seems to be highly influenced by Semas and its receptors.

Nevertheless, more and more investigations are elucidating new effects of semaphorins during other pathological processes. The first step in research leading to the suggestion that semaphorins might play a role in angiogenesis and/or vascular permeability was the fact that VEGF, a renowned angiogenic and vascular permeability factors shares the same receptor with semaphorins, the membrane bound receptor Nrp. Indeed, studies in genetically modified animals, have demonstrated the importance of Nrp signaling during angiogenesis. The overexpression or absence of Nrp1 results in an abnormal cardio-vascular system (Kawasaki, et al., 1999, Kitsukawa, Shimono, Kawakami, Kondoh and Fujisawa, 1995).

Nrp1 was identified as a specific receptor for VEGF-165, and studies have suggested injury induced effects of neuropilins, VEGF and class 3 Semas in neovascularization in and around CNS lesion area (Beggs and Waggener, 1979, Imperato-Kalmar, et al., 1997). Already in year 1999, it was known that Nrp1 expression is induced on the surface of blood vessels during neural scar formation following injury (Pasterkamp, Giger, Ruitenberg, Holtmaat, De Wit, De Winter and Verhaagen, 1999). However, Nrp1 seems not to be only involved in angiogenesis, but also in enhanced vascular permeability, as suggested by Kitsukawa and colleagues (Kitsukawa, Shimono, Kawakami, Kondoh and Fujisawa, 1995). Studies in diabetic retinopathy have demonstrated that Sema3A is an early inducer of vascular permeability via Nrp1 and even propose that Sema3A might represent an attractive alternative therapeutic strategy during diabetic retinopathy (Cerani, et al., 2013).

Therefore, Semas and Nrp seem to play an important role during angiogenesis and vascular permeability and might be involved in these processes during endometriosis.

1.10 Thesis approach

In this study, analysis of human tissue, peritoneal fluid and serum of patients with endometriosis were performed. Furthermore, in vitro analysis with peritoneal fluid aimed to elucidate possible effects of the peritoneal fluid on specific cells.

The emphasis of the study laid on the chronic inflammatory condition during endometriosis and the unsolved pain emergence. Therefore, the sympathetic nervous system and the potential role of specific Semas in the modulation of the peritoneal sympathetic innervation of endometriosis patients was analyzed. Here, it was important to verify the appearance of nerve fibers and their possible regulators, the nerve repellent factors “semaphorin” and their main receptors, which potentially also modulate vascular permeability. Furthermore, the pro- and anti-inflammatory factors balance in women with endometriosis was analyzed.

In tissue it was important to determine if the overall peritoneum is affected or if the peritoneum is only locally affected by the endometriosis lesions. Therefore, tissue samples of endometriosis patients with macroscopic visible lesions (affected) and tissue samples of endometriosis patients with no macroscopic visible lesions (unaffected peritoneum) were analyzed.

In matter of Semas and their receptors, it was important to analyze the exact pattern of expression in tissue, since Semas act locally. Furthermore, the expression of Semas was characterized, to elucidate which type of cells and/or structures secrete semaphorins. The expression of Sema receptors was analyzed to determine if Semas action potentially affects innervatory system or vascularization/vascular permeability during endometriosis.

The peritoneal fluid is in constant contact with the peritoneum and might therefore affect the peritoneum; so it was necessary to determine the expression of Semas and receptors in the fluid. In addition, the inflammatory status was analyzed by determining the occurrence of important pro- and anti-inflammatory cytokines.

After these conditions were elucidated in samples from patients, we aimed to verify the effects of peritoneal fluid in vitro. Peritoneal fluid neurotrophic properties were analyzed. Additionally, the ability of peritoneal fluid to induce Semas expression in fibroblasts was analyzed.

Altogether, this work aimed to elucidate a correlation between immune response and innervation during endometriosis, consequently suggesting important factors, which potentially support and maintain the chronic inflammatory condition in endometriosis and therefore might play a role in pain emergence.

2 Materials and Methods

2.1 Equipment

	Company and Model
Cameras	Canon powershot G5, Canon GmbH (Krefeld)
Centrifuges	Ultracentrifuge Optima L-90K and L-90L, Heraeus Instruments (Hanau, Germany)
Coolplate	Tissue cool plate COP 20, Medite Medizintechnik (Burgdorf, Germany)
Fluorescence lamp	Illuminator HXP120C, Zeiss (Jena, Germany)
Gel chambers	Gel chambers, Peqlab (Erlangen, Germany)
Incubator	Incubator BBD 6220, Heraeus (Hanau, Germany)
	Incubator model 3000, Jane Schütz GmbH (Hammelburg, Germany)
Magnetic stirrer	KMO 2 basic, IKA Werke GmbH (Staufen, Germany)
Microscopes	Binocular Wild M8, Leica Biosystems GmbH (Nussloch)
	Axiophot, Zeiss (Göttingen, Germany)
	Zeiss LSM META
Microtome	HM 400R, MICROM GmbH (Walldorf, Germany)
Microwave	NN-E205W, Panasonic (Munich, Germany)
PCR System	StepOne Real-Time PCR System, Life Technologies (Darmstadt, Germany)
pH Meter	pH 300, Hanna Instruments (Kehl am Rhein, Germany)
Pipettes	Pipetteman P10/ 20/ 200/ 1000, Gilson (Middleton, USA)
Pipetting unit	AccuJet Pro, Brand (Wertheim, Germany)

Power units	Power Supply Pac 3000, Bio-Rad Laboratories (Munich, Germany)
Shaker	Nutation mixer, VWR (Darmstadt, Germany)
Steam cooker	Steam cooker, Braun (Kronberg, Germany)
Sterile hood	Hera Safe, Heraeus Instruments (Hanau, Germany)
Spectrophotometer	ND-100, Peqlab (Erlangen, Germany)
Thermal Cycler	DNA engine PTC-200, Biorad (München, Germany)
Thermal Shaker	Thermomixer comfort, Eppendorf (Hamburg, Germany)
Tweezers	Dumont&Fils (Montignez, Switzerland)
	Carl Roth GmbH (Karlsruhe, Germany)
Vortexer	Vortex Genie 2, Scientific Industries (Bohemia, USA)
Waterbath	WB Typ1012/1013, Medax GmbH (Neumünster, Germany)

2.2 Consumable supplies

	Company and Model
Autoclave tape	SteriClin sticky tape, VP Group (Feuchtwangen, Germany)
Microtome blades	Disposable Microtome blades, Feather (Osaka, Japan)
Cleaning wipes	Kimtech precision wipes, Kimberley & Clark (Roswell, USA)
Cover glasses	Microscope cover glasses (40mm/ 60mm), Labomedic (Bonn, Germany)
Cover slips	Hybrislips (60mm), Sigma Aldrich (St. Louis, USA)
Embedding cassettes	Histosette embedding cassettes, VWR (Darmstadt, Germany)
Filter tips	ART 100/ 200/ 1000 barrier tips, Fisher Scientific (Waltham, USA)

Gloves	Dermaclean latex gloves, Ansell Healthcare (Bonn, Germany)
	Nitratex nitril gloves, Ansell Healthcare (Bonn, Germany)
Maxiprep kit	Purelink Maxi-prep kit, Invitrogen (Carlsbad, USA)
Microscope slides	Superfrost/ Superfrost Ultra Plus, Menzel-Gläser (Braunschweig, Germany)
Microtiter plates	Falcon 6/ 12/ 24/ 96 well plates, BD Biosciences (Heidelberg, Germany)
Parafilm	Laboratory film 'M', Pechiney Plastic Packaging (Chicago, USA)
Pasteur pipettes	Plastic pipettes 3mL/ 5mL, RatioLab (Dreieich, Germany)
PCR plates	MicroAmp 48-Well plate, Applied Biosystems (Darmstadt, Germany)
PCR tubes	PCR strip tubes 0,2mL, VWR (Darmstadt, Germany)
Petri dishes	Falcon petri dishes (15mm), BD Biosciences (Heidelberg, Germany)
Pipette tips	Gilson pipette tips (0.5-20 µL, 21-200 µL, 201-1000 µL), Greiner Bio-One (Frickenhausen, Germany)
Reagent tubes	Eppendorf tubes (0,5mL; 1,5mL; 2mL), Eppendorf (Hamburg, Germany)
	Falcon conical tubes (15mL; 50mL), BD Biosciences (Heidelberg, Germany)
Serological pipettes	Costar plastic serological pipettes, Sigma Aldrich (St. Louis, USA)
Slides boxes	Micro slide box (plastic), VWR (Darmstadt, Germany)

2.3 Reagents and Chemicals

	Company
Acetic anhydride (Ac2O)	VWR International (Darmstadt, Germany)
Acetone	Roth (Karlsruhe, Germany)
Agarose (Ultrapure)	Invitrogen (Carlsbad, USA)
Albumin Bovine Serum (BSA)	Sigma Aldrich (St. Louis, USA)
Ampicillin	VWR International (Darmstadt, Germany)
Antibody Diluent	ABD, Dako (Hamburg, Germany)
Anti-DIG-AP Fab fragments	Roche Applied Science (Penzberg, Germany)
Aqua-PolyMount	Polysciences Inc. (Eppelheim, Germany)
Avidin/Biotin Blocking system	Dako (Hamburg, Germany)
BM purple	Roche Applied Science (Penzberg, Germany)
Bovine serum albumin	BSA, Biochrom (Berlin, Germany)
Bromochloropropane	BMC, Sigma Aldrich (Steinheim, Germany)
Bromphenol blue	Sigma Aldrich (Steinheim, Germany)
Chloroform (CHCl3)	VWR International (Darmstadt, Germany)
Collagen IV	Biochrom AG (Berlin, Germany)
Culture medium (LB)	AppliChem (Darmstadt, Germany)
DAB Kit	DAB chromogen and substrate buffer, Dako (Hamburg, Germany)
Dako pen	Fat-pen, Dako (Hambrug, Germany)
ddH20	Ampuwa, Fresenius (Bad Homburg)
dH20	MilliQ, Millipore (Billerica, USA)
Deoxycholate	AppliChem (Darmstadt, Germany)
Dextran sulfate	AppliChem (Darmstadt, Germany)

Digoxigenin-labeled NTPs	Roche Applied Science (Penzberg, Germany)
Disodium phosphate (Na2HPO4)	VWR International (Darmstadt, Germany)
DNA ladder	1kb plus, Invitrogen Corporation (Carlsbad, USA)
DNA loading Dye	6x loading Dye, Thermo Scientific (Schwerte, Germany)
dNTPs	Peqlab (Erlangen, Germany)
Dulbecco´s Modified Eagle´s Medium	DMEM, Gibco (Karlsruhe, Germany)
Dubelcco´s PBS	PAA Laboratories GmbH, (Linz, Austria)
Eosin Solution	Sigma Aldrich (St. Louis, USA)
Estradiol	17-β estradiol, Sigma Aldrich (Steinheim, Germany)
Ethidium bromide (EtBr)	Invitrogen Corporation (Carlsbad, USA)
Ethanol (EtOH)	VWR International (Darmstadt, Germany)
Ethylenediaminetetraacetic acid (EDTA)	VWR International (Darmstadt, Germany)
FastRED chromogen system	Biolegend Covance (San Diego, USA)
Ferricyanide	AppliChem (Darmstadt, Germany)
Ferrocyanide	AppliChem (Darmstadt, Germany)
Ficoll	Sigma Aldrich (St. Louis, USA)
Fluoromount	Dapi Fluoromount G, Southern Biotechnology (Birmingham, USA)
Formamide	Invitrogen Corporation (Carlsbad, USA)
Glycerol	Fisher Scientific (Schwerte, Germany)
Glycine	VWR International (Darmstadt, Germany)
Hydrochloric acid (HCl)	VWR International (Darmstadt, Germany)
Hydrophobic barrier pen	Dako Pen, Dako (Hamburg, Germany)

Hematoxylin	Sigma Aldrich (St. Louis, USA)
Igepal	Sigma Aldrich (St. Louis, USA)
Isopropyl alcohol (IPA)	VWR International (Darmstadt, Germany)
Kaiser's glycerol gelatin	Merck Millipore (Darmstadt, Germany)
Levamisole	Sigma Aldrich (St. Louis, USA)
Lithium chloride (LiCl)	Sigma Aldrich (St. Louis, USA)
Magnesium chloride (MgCl2)	VWR International (Darmstadt, Germany)
Nerve growth factor 2.5S	NGF 2.5S, Sigma Aldrich (Steinheim, Germany)
Normal goat serum (NGS)	Sigma Aldrich (St. Louis, USA)
Nuclease free water	Life Technologies (Darmstadt, Germany)
Paraffin	McCormick Scientific (Richmond, USA)
Paraformaldehyde (PFA)	VWR International (Darmstadt, Germany)
PCR rxn buffer (10x)	Invitrogen Corporation (Carlsbad, USA)
Phenol	AppliChem (Darmstadt, Germany)
Phenol-Chloroform	AppliChem (Darmstadt, Germany)
RNA Polymerase buffer (10x)	Invitrogen Corporation (Carlsbad, USA)
Peroxidase Blocking Reagent	Dako (Hamburg, Germany)
PolyMount	Polysciences Inc. (Eppelheim, Germany)
Polysorbate 20 (Tween 20)	VWR International (Darmstadt, Germany)
Potassium chloride (KCl)	VWR International (Darmstadt, Germany)
Restriction enzymes	New England Biolabs (Ipswich, USA)
	Roche Applied Science (Penzberg, Germany)
RNase inhibitor	Roche Applied Science (Penzberg, Germany)
Sarcosyl	Sigma Aldrich (St. Louis, USA)
Semaphorin 3C and 3F	Recombinant semaphorin Fc Chimera (truncated), R&D Systems (Minneapolis, USA)

Sodium acetate (NaAc)	Merck (Darmstadt, Germany)
Sodium citrate	VWR International (Darmstadt, Germany)
Sodium chloride	VWR International (Darmstadt, Germany)
Sodium diphosphate	VWR International (Darmstadt, Germany)
Sodium hydroxide (NaOH)	VWR International (Darmstadt, Germany)
Sodium tetraborate decahydrate	VWR International (Darmstadt, Germany)
Streptavidin-AP	Streptavidin alkaline phosphatase, Roche Diagnostics (Penzberg, Germany)
Streptavidin-HRP	Streptavidin horseradish peroxidase, Dako (Hamburg, Germany)
Sucrose	Sigma Aldrich (St. Louis, USA)
Transforming growth factor	TGFβ, Sigma Aldrich (St. Louis, USA)
Transcription buffer	Roche Applied Science (Penzberg, Germany)
Triethanolamine (TEA)	VWR International (Darmstadt, Germany)
Tris-aminomethane (TRIS)	Merck (Darmstadt, Germany)
Triton X-100	Merck (Darmstadt, Germany)
Trypsin	Sigma Aldrich (St. Louis, USA)
Xylol	Arcos Organics (Geel, Belgium)

2.4 Solutions and Buffers

Buffer/ Solution	**Ingredients**
Tris-buffer (10x)	68.5 g Tris-HCl (10mM)
	8 g Tris-Base
	87.8 g NaCl (150 mM)
	fill up to 1000 ml with aqua dest

	and adjust pH 7.4-7.6 with HCl/ NaOH
Citrate buffer (10mM, pH 6)	18 ml solution A
	82 ml solution B
	fill up to 1000 ml with aqua dest
Solution A	21.01 g citric acid
	fill up to 1000 ml with aqua dest
Solution B	29.41 g sodium citrate
	fill up to 1000 ml with aqua dest
Denhardts (100x)	5 g Ficoll
	5 g PVP
	5 g BSA
	250 mL dH2O
Hybridization Solution	50 mL Formamide (deionized) (50%)
	20 mL 50% Dextran sulfate
	1 mL 100 x Denhardt's (10%)
	2.5 mL yeast tRNA (10mg/mL) (10%)
	6 mL 5M NaCl (0.3M)
	2 mL 1M Tris-HCl, pH8 (20mM)
	1 mL 0.5M EDTA (5mM)
	1 mL 1M NaPO4, pH8
	5 mL 20% Sarcosyl (10mM)
	11.5 mL DEPC-H2O (1%)
NTMT	2 mL 5M NaCl (100mM)
	10 mL 1M Tris-HCl, pH9.5 (100mM)
	5 mL 1M MgCl2 (50mM)
	0.1 mL Tween 20 (0.1%)

	82.9 mL dH2O
PBS (5x)	40 g NaCl (137mM) 1 g KCl (2,7mM) 7.1 g Na2HPO4 (10mM) 1.36 g KH2P4 (2mM) in 1 L dH2O
PBT	10 mL 10% Tween-X (0.1%) 990 mL 1 x PBS PFA (20%) 500 g PFA 2.0 L ddH2O 8.0 mL NaOH
RNase Buffer	100 mL 5M NaCl (0.5M) 10 mL 1M Tris-HCl, pH7.5 (10mM) 10 mL 0.5M EDTA, pH8 (5mM) 880 mL dH2O
RNAseA	100 mg RNAseA 100 µl Tris-HCL, pH7.5 30 µl 5M NaCl (15mM) 9.8 ml dH2O
SSC (20x)	88.2 g NaCitrate (C6 H5 Na3 O7) 174 g NaCL in 1 L dH2O pH adjusted to 7.0
TAE (50x) (Gel running buffer)	242 g Tris-base 57.1 mL Glacial acetic acid 100 mL 0,5M EDTA, pH8.0
Triethanolamine-HCl	18.6 g Triethanolamine

4.5 mL 10M NaOH (45mM)

2.6 mL 12M HCl (31mM)

in 1 L dH2O

2.5 Enzymes

Polymerases	Company
Taq DNA Polymerase	GE Healthcare (Buckinghamshire, UK)
Taq RNA T3 Polymerase	Roche Applied Science (Penzberg, Germany)
Taq RNA T7 Polymerase	Roche Applied Science (Penzberg, Germany)

Restriction Enzyme	Buffer	Company
EcoRI	NEB 1-4	Roche Applied Science (Penzberg, Germany)
HindIII	Tango	Roche Applied Science (Penzberg, Germany)
NotI	Buffer O	New England Biolabs (Ipswich, USA)
SacI	Tango	New England Biolabs (Ipswich, USA)
XhoI	Buffer O	Roche Applied Science (Penzberg, Germany)

Other Enzymes	Company
Deoxyribonuclease (DNAse)	Promega Corp. (Fitchburg, USA)
Proteinase K (ProtK)	Roche Applied Science (Penzberg, Germany)
Ribonuclease (RNAse A)	Invitrogen Corporation (Carlsbad, USA)

2.6 Bacteria

Escherichia coli DH5α competent cells were used for amplifying plasmid DNA.

2.7 Molecular methods

2.7.1 RNA isolation from cells

Cells RNA isolation was performed with the chloroform method. Cells were washed with PBS and 1ml Trizol was added for ca. 3 minutes to loose the cells form the well plate. Subsequently, 200 µL of chilled chloroform is added and vigorously shaked for 15 seconds. The samples were then chilled for 5 minutes at room temperature. Afterwards, centrifugation at 13000 rpm for 30 minutes at 4°C followed. The upper layer was transferred into a fresh tube and 500 µL cold isopropanol (-20°C) was added. Again probes were incubated at room temperature for 10 minutes and then centrifuged at 13000 rpm for 30 minutes at 4°C (pellet formation). Supernatant was discarded and 1 mL 75% cold ethanol (-20°C) was added to wash the pellet. The probes were then centrifuged again at 13000 rpm for 30 minutes at 4°C, the supernatant was removed and the probes were centrifuged again under the same conditions but only for 10 seconds. The supernatant was then removed and the pellet was air-dried. The dry pellet was dissolved in 20 µL RNase free water and the RNA concentration was measured at the Nanodrop.

2.7.2 cDNA synthesis

RNA samples obtained from cells were transcribed in cDNA for PCR with the high capacity cDNA reverse transcription kit from Applied Biosystems, Germany. A total amount of 0.5 µg RNA (diluted in 10 µL) were used for this aim.

Sample Solution	**cDNA (+)**	**cDNA (-)**
Nuclease free H2O	3.80 µL	8.80 µL
10x Reverse transcription buffer	2.00 µL	2.00 µL
100 mM dNTPs	0.20 µL	0.20 µL
10x random primer	2.00 µL	2.00 µL
MultiScribe™ Reverse Transcriptase (50U)	1.00 µL	0.00 µL

RNAse inhibitor (20U)	1.00 µL	0.00 µL
Solution (total)	10.00 µL	10.00 µL

10 µL of each RNA sample was pipetted into each tube and then 10 µL of the mix was added on each sample for cDNA (+) and cDNA (-), which was used as a negative control for the PCRs. Consequently, the samples were transcribed in the programmable thermal controller. Thermal profile:

25°C 10 minutes

37°C 120 minutes

85°C 5 minutes

4°C unlimited

The probes were then diluted 1:5 by adding 80 µL nuclease free H2O to the 20 µL samples. Probes were ready for PCR and were stored at -20°C.

2.7.3 Polymerase chain reaction (PCR)

PCR is a technique used to amplify the DNA (deoxyribonucleic acid) fragments. This in vitro method relies on the enzymatic replication of the DNA with the help of a DNA Polymerase and several heating and cooling cycles. After the denaturing of double-stranded DNA at 94°C, oligonucleotides (primers) are able to bind to a strand of the single-stranded DNA during the annealing cycles (usually 54°C to 64°C) and the thermo-stable DNA-polymerase (e.g. Taq Polymerase) is able to synthesize new strands of DNA (at 72°C). The DNA itself is used as a template for further amplification in every cycle generating a chain reaction in which the DNA is amplified exponentially. In this study, the PCR was used to determine expression levels of different genes of interest in fibroblasts after various treatments.

2.7.4 Oligonucleotides

The applied oligodeoxyribonucleotides were obtained from Metabion, Germany.

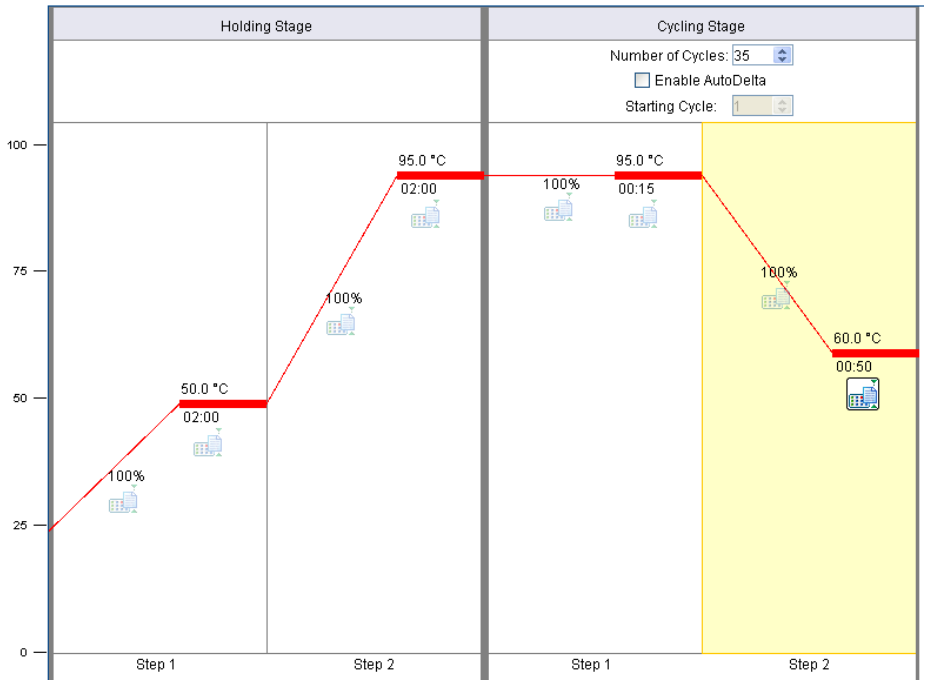
Primer	Sequence
Sema3C-forwards	5'-CACCTTgTATgTCTgTgggAgTg-3'
Sema3C-reverse	5'-TCgATCATgAATACCTggTCCT-3'
Sema3F-forwards	5'-ACAACCCCATgTgCACCTAT-3'
Sema3F-reverse	5'-gCCTTTTCCTgACTCCAgTTT-3'
Nrp2-forwards	5'-gATCATCCTACAgTTCCTgACCTT-3'
Nrp2-reverse	5'-gCCAATCAgAggTCCAACAT-3'

2.7.5 PCR mix and program

PCR-Sample Solution	final conc.	Volume
2xexpress supermix	1x	7.50 µL
Forward primer	200 nM	0.15 µL
Reverse primer	200 nM	0.15 µL
Library probe	100 nM	0.15 µL
18s housekeeping gene (primer 3µM, probe 5µM)	24 nM/40 nM	0.12 µL
dH2O		5.43 µL
cDNA		1.50 µL
Solution (total)		15.00 µL

Library probes were obtained from Roche, Germany. Utilized probes: #26 probe for Sema3C, #1 probe for Sema3F and Nrp2. 18s probe is VIC labeled and was obtained from Applied Biosystems, Germany.

PCR program:

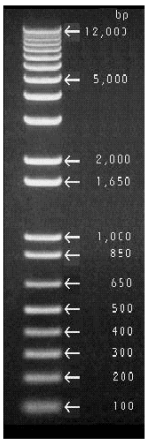


2.7.6 Agarose gelelectrophoresis

Gelelectrophoresis is an analytical method used to separate different kinds of molecules (e.g. DNA) according to their size and electric charge. An electric field is generated, which passes through the gel loaded with the different molecules. Consequently, negatively charged molecules will move towards the positively charged anode and the positively charged molecules will move towards the negatively charged cathode. The density of the gel influences the movement of the molecules, since it is a porous matrix, which acts as a sieve, letting smaller molecules pass easier. Depending on the concentration of the agarose, the gel is more or less dense. Following the separation by gelelectrophoresis, the size of the molecules can be determined due to the molecular weight standards, which serve as markers for the size of the DNA fragments.

In this study gelelectrophoresis was performed to determine the size of PCR fragments, to check for the digestion of plasmids and to verify the proper size and concentration of RNA *in situ* probes. Furthermore, it was used to verify that the products obtained after PCR are the genes of interest.

Issue	gel conc.	EtBr	Voltage	Time	Marker
DNA (200-600 bp)	1.5%	5 µg/ml	100	~ 40 min	1Kb
Plasmids (1-3.5 kb)	1.0%	5µg/ml	120	~60 min	1Kb
RNA (300-2000 bp)	1.5%	5µg/ml	120	~45 min	1Kb



1 Kb PLUS DNA LADDER
0.7 µg/lane
0.9% agarose gel

2.7.7 Transformation

The transformation of E.coli DH5α with plasmid DNA was performed with the heat shock method. For our study, chemical competent cells and plasmid DNA with ampicillin resistance were used. To achieve transformation of the bacteria, the plasmid DNA was added to the chemical competent cells and incubated for 5 minutes. Then the cells were heat-shocked at 42°C for 30 seconds. During the heat shock, the membrane of the E.coli cells becomes porous and the plasmid DNA has the opportunity to enter into the cell. Immediately after the heat shock, the bacteria were transferred into ice and incubated for a few minutes. This helps the cells recovering. After that, cells were plated on LB agar plates containing 20µl/ml ampicillin and were grown overnight at 37°C. Only bacteria containing the plasmid DNA with the ampicillin resistance will grow and form colonies.

2.7.8 Maxi-prep

Maxi-preps are used to produce high yields of plasmid DNA. In this study, high yields of plasmid DNA were needed to make RNA probes, for RNA *in situ* hybridization.

Before performing a Maxi-prep, one colony of the transformed E.coli bacteria was picked and grown for approximately 5 hours in 10 ml LB medium with 20 µl/ml ampicillin in a 37°C incubator-shaker. This culture was then transferred into an Erlenmeyer flask containing 100 ml LB medium plus 20µl/ml ampicillin and incubated in a shaker at 37°C overnight. The isolation of the plasmids was performed via silica gel columns (Maxi-prep Kit, Invitrogen order number). Further steps of the isolation were implemented as specified by the manufacturer. At the end of the isolation procedure, the concentration of the DNA was measured with the Nanodrop Spectrophotometer. Plasmid DNA was stored at 4°C.

2.7.9 Plasmid digestion

Plasmids consist of a backbone that allows replication and contain an antibiotic resistance gene and a multiple cloning sites (Evans and McShane). DNA inserts (e.g. specific cDNA or fragments of a cDNA) can be cloned into the MCS. For the digestion, it is necessary to use restrictions enzymes, which are able to cut the plasmid at specific restriction sides. Depending on how many restriction sites are present for a given restriction enzyme or how many restriction enzymes are used, the plasmid DNA either gets linearized or DNA inserts can be cut out of the plasmid. For this study, we wanted to linearize the plasmid DNA for the preparation of RNA probes for in situ hybridization. Therefore we used restriction enzymes with only one restriction site in the plasmid DNA.

Plasmid digestion was performed in a total volume of 100 µL:

20 µg Plasmid

10 µL 10 x NEB1-4 Buffer

1 µL 100 x BSA

5 µL Enzyme

55 µL dH2O

The reaction mix was incubated for 3 hours at 37°C. Afterwards, gelelectrophoresis was performed with 5 µL of the reaction mix to verify that the plasmid was fully digested; the uncut plasmid was used as a control. If the plasmid was not fully digested,

additional incubation at 37°C was necessary. If the plasmid was fully digested, the DNA was purified. The linearized plasmid DNA was stored at -20°C.

2.7.10 Purification of the linearized plasmid DNA

Different methods can be used for purification of DNA. In this study, Phenol-Chloroform extraction was used to separate the DNA from the other components of the digestion mix (enzymes, salts, etc.) after plasmid digestion.

Phenol-Chloroform extraction with EtOH precipitation:

To 100 µl of the reaction mix 1 volume (100 µl) of phenol-chlorophorm was added and vortexed. Subsequently, the samples were centrifuged at 14000rpm for 10 minutes to separate the aqueous and phenol/chloroform phase. The upper, aqueous phase was transferred to a new tube and 0.3 M sodium acetate (pH 5.2) and 2 volumes of ice cold EtOH were added. The sample was mixed by vortexing and incubated for 30 minutes at -80 °C for DNA precipitation. To recover the DNA precipitate, the sample was centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was carefully removed and was washed with 70% EtOH. After an additional centrifugation and removal of the 70% EtOH, the pellet was air-dried at room temperature for 5 minutes or more. Afterwards the pellet was resuspended in 20 µl RNase-free dH2O.

2.7.11 Transcription of RNA probes

In vitro transcription of RNA probes requires a purified and linearized DNA template that contains recognition sites for RNA polymerases (T3, T7, SP6), which transcribe the RNA from the DNA template. A transcription buffer that contains Dithiothreitol (DTT) and magnesium ions, each important for avoiding DNA dimerization and contributing to the elongation of the RNA is also required. Since the RNA probes for in situ hybridization have to hybridize to messenger RNA, complementary antisense transcripts are required. The ribonucleotide-triphosphates applied for the transcription included DIG-labeled UTP´s (Digoxigenin-11-uridine-5'-triphosphate), which are recognized by anti-DIG-AP Fab fragments. The alkaline phosphatase uses BM purple as a substrate and results in a purple precipitate.

Protocol for *in vitro* transcription:

1. Mix 1.5 µL Purified DNA (1-2 µg)

2.0 µL (10x) Transcription Buffer

2.0 µL (10x) DIG-NTP labeling mix

0.5 µL RNase inhibitor

1.5 µL RNA polymerase

12.5 µL ddH2O

20.0 µl total

2. Incubate mix at 37°C for 2:30 hours (transcription).

3. Add 1µl DNase and incubate for 15 minutes (removal of DNA template).

4. Add 2 µL of EDTA (4mM), 2.5 µL LiCl (100mM) and 75 mL 100% EtOH and incubate at -80°C for 15min (RNA precipitation).

5. Centrifuge suspension at 13 000 rpm for 15 minutes at RT and remove supernatant (RNA in pellet).

6. Wash with 70% EtOH.

7. Centrifuge at 13 000 rpm for 10 minutes and remove supernatant (RNA again in pellet).

8. Air-dry pellet at RT for 3-5min.

9. Take up pellet in 50 µL ddH2O + 1% RNase inhibitor.

The RNA concentration was measured with Nanodrop and the RNA probe was stored at -20°C.

2.8 Patient collective and processing of samples

2.8.1 Peritoneal tissue

Peritoneal tissue samples of 38 patients with clinical and histological proven endometriosis were collected during laparoscopy. The patients had a regular menstrual cycle and were not under hormonal treatment at least three months prior surgery, excluding eight patients which used oral contraceptives. The menstrual cycle phase of the women by the day of laparoscopy was proliferative in four cases, secretory in seven cases and menstrual in one case, in 18 cases the cycle phase was not declared. Only women in reproductive age were included in this study (range: 19-

53 years, mean: 32.8). The stage of endometriosis was provided during the surgery according to the revised American Society of Reproductive Medicine (rASRM) (Medicine, 1997) (rASRM I= 6, II= 12, III= 5, IV= 7, not known= 8) (Table 1). Furthermore, we collected unaffected peritoneal tissue samples of 24 patients with endometriosis of regions with no macroscopic or histological prove of endometriosis lesions.

As control group, 10 healthy peritonea from women with macroscopic and histological proven exclusion of endometriosis were collected during hysterectomy (uterine fibroids). The tissue probes comprised peritoneal tissue and the underlying subperitoneal fat. The patients had a regular cycle. The mean age of the women in the control group was 41.3 (range 30-50 years).

All patients gave their consent and signed for the participation in this study and the Ethics Committees of the Charité – Universitätsmedizin Berlin approved this study with the ethic vote EA4/023/05.

2.8.1.1 Paraffin embedding

The tissue was collected during laparoscopy. Paraffin embedding solidifies biological sample and allows the preparation of thin sections (2 µm). This requires an elaborated preparation of the sample. The tissue has to be thoroughly fixed in 4% PFA to maintain its normal morphology. Afterwards the tissue was dehydrated in ascending concentrations of ethanol (70% - 100% EtOH), followed by incubation in Xylol (an intermediate medium) to prepare the tissue to absorb the paraffin The tissue was then incubated for 3 times in changing baths of paraffin at 60°C to remove traces of Xylol. The next step was the actual embedding of the tissue; it was transferred into a mold and filled up with fresh paraffin (~60°C). Subsequently the paraffin blocks were kept at room temperature (RT) and hardened. After that, the blocks could be sectioned with a microtome. Storage of the blocks was possible at RT for unlimited time.

2.8.1.2 Tissue sectioning

The paraffin embedded tissue was sectioned using a microtome. For this purpose the paraffin blocks were positioned on a plastic cassette and attached using liquid paraffin

(60°C). The cassette was then clamped in the microtome and trimmed until tissue was visible. Subsequently the specimen was sectioned. The slice thickness was 2 µm. After cutting, the slices were transferred into a water bath (~38°C), to straighten the section. To take up the sections, fifteen superfrost ultra plus slides were used. Two sections were then placed consecutively on each one. In such a way, every slide had sections form different levels of the sectioned tissue. This procedure was repeated until the slides were completely filled with sections or until the tissue was entirely sectioned. The sectioned tissue on the slides was dried overnight at 37°C, where the sections flattened. Slides were stored at RT.

2.8.2 Peritoneal fluid

Peritoneal fluid of patients with proven endometriosis and without endometriosis was collected at the beginning of the surgery. Consequently, the probes were centrifuged at 3000 rpm for 5 minutes and the upper layer was transferred into a tube in which it was frozen in liquid nitrogen and stored at -80°C. Only clear and not diluted fluids were utilized for the experiments.

Volume of peritoneal fluid varies from women to women and the possible amount that can be collected is not very high. Therefore, unfortunately not always the same probes could be used for all experiments. Samples utilized for ELISA are listed in section 2.13.

2.8.3 Serum

Serum form patients with proven endometriosis and without endometriosis were collected in BD SST II Advance tubes with gel. Tubes were gently mixed 5-6 times, then chilled for 30 minutes. Consequently, the tubes were centrifuged at 3000 g for 5 minutes. The upper layer was stored at -20°C.

The serum probes used for the experiments are listed in section 2.13.

2.9 Hematoxylin and Eosin (H&E) staining

Hematoxylin and Eosin (H&E) staining is a commonly used method to stain the cytoplasm and nuclei of cells in tissue sections to allow the analysis of the histological

properties of tissue sections. Hematoxylin stains the nuclei in dark blue and Eosin colors the cytoplasm and extracellular matrix in light pink. During this study, H&E staining was used for histological analysis of tissue sections. Paraffin sections were dewaxed and rehydrated before staining. The slides with the paraffin sections were washed for 2min in ddH2O. Then, they were stained in Hematoxylin for 3min and rinsed under tap water for 2-5 min. Next, the slides were transferred to 75% EtOH-HCl (6 µL/mL) for 30 s for fixation of the staining and washed under tap water for 5 min. Afterwards, the slides were washed in ddH2O and stained in Eosin for 3min. This was followed by dehydration: 3min 70% EtOH, 3min 95% EtOH (2 x), 3min 100% EtOH (2 x), 3min Xylol (3 x). The stained sections were then embedded in PolyMount, a Xylene based mounting-medium and stored at RT.

2.10 Immunohistochemical stainings

Immunohistochemical stainings (IHC) are used to detect proteins of interest on tissue sections or cells. For detection, a specific antibody against the protein of interest is required. To perform IHC on paraffin sections, sections had to be dewaxed and rehydrated before the IHC procedure.

First, the sections were washed with Tris. Then, sections were incubated for 15 minutes in Target buffer in the steam cooker, in order to expose the antigen-binding site. Subsequently the sections were cooled down on ice for ca. 20 minutes. Upon this, antibody in specific concentrations were applied. Biotinylated secondary antibodies were used for detection of the first antibodies. To detect the biotin signal, streptavidin-alkaline phosphatase (Dilution 1:1000, Roche, Germany) was added and as chromogen FastRed (Dako, Germany) or streptavidin-horse radish peroxidase (Dilution 1:400, Dako, Germany) was added and then the chromogen DAB (Invitrogen, Germany). All antibodies and streptavidin were applied and incubated for 45 minutes on the tissue sample. FastRed (red staining) was incubated for 10-15 minutes and DAB (brown staining) for 5 minutes.

Antibody	Dilution	Company
First:		
Monoclonal mouse anti-TH	1:250	Abcam, UK
Polyclonal rabbit anti-Sema3C	1:150	Sigma-Aldrich, Germany
Polyclonal goat anti-Sema3F	1:300	Sigma-Aldrich, Germany
Monoclonal mouse anti-Nrp1	1:100	Santa Cruz Biotech, USA
Monoclonal mouse anti-Nrp2	1:250	Santa Cruz Biotech, USA
Polyclonal mouse anti-PlxnA3	1:200	Millipore, USA
Polyclonal rabbit anti-PlxnA4	1:200	Sigma-Aldrich, Germany
Secondary:		
Rabbit anti mouse IgG	1:400	Jackson ResearchLabs, Germany
Mouse anti-rabbit IgG	1:400	Jackson ResearchLabs, Germany
Rabbit anti-rat IgG	1:400	Dako, Germany
Rabbit anti-goat IgG	1:400	Dako, Germany

2.11 Immunofluorescence double staining

For the immunofluorescence double stainings we used following antibodies:

Antibody	Dilution	Company
First:		
Monoclonal mouse anti-CD68	1:25000	Dianova, Germany
Polyclonal rabbit anti-Sema3C	1:150	Sigma-Aldrich, Germany
Polyclonal goat anti-Sema3F	1:300	Sigma-Aldrich, Germany
Monoclonal mouse anti-Nrp1	1:100	Santa Cruz Biotech, USA
Monoclonal mouse anti-Nrp2	1:250	Santa Cruz Biotech, USA
Polyclonal mouse anti-PlxnA3	1:200	Millipore, USA
Polyclonal rabbit anti-PlxnA4	1:200	Sigma-Aldrich, Germany

Polyclonal rabbit anti-P4HB	1:1000	Sigma-Aldrich, Germany
Polyclonal goat anti-TH	1:100	Abcam, UK

Antibody	**Dilution**	**Company**
Secondary:		
Donkey anti mouse Alexa 488	1:100	Abcam, UK
Donkey anti rabbit Alexa 488	1:100	Abcam, UK
Donkey anti mouse Dylight555	1:100	Biomol, Germany
Donkey anti goat Dylight555	1:100	Biomol, Germany
Donkey anti rabbit Dylight555	1:100	Biomol, Germany

We analyzed the co-expression of Nrp1, Nrp2, PlxnA3 and PlxnA4 with TH-positive nerve fibers and evaluated the expression of Sema 3C/3F in P4HB-positive fibroblasts and CD68-postive macrophages. Nuclear counterstain was performed with Dapi (4',6-diamidino-2-phenylindole). Gut sections served as positive control for P4HB and TH staining, spleen sections for the macrophages staining. For the negative control, the primary antibody was excluded during the staining.

2.12 In situ Hybridization (ISH)

RNA *in situ* hybridization is used to detect ribonucleic acid on tissue sections or in cells. Specifically, it is used to detect the presence of the messenger (m)RNA of the genes of interest and allows therefore to monitor gene expression. For hybridization with the mRNA complementary labeled anti-sense probe (riboprobe) of the sequence of the RNA of interest is generated, so that the labeled riboprobe hybridizes with the complementary mRNA. This labeled RNA complex can be visualized by immunohistochemistry. To perform ISH on paraffin sections, sections had to be dewaxed and rehydrated before the ISH procedure.

First, the sections were post-fixed in 4% paraformaldehyde (PFA) for 10 min followed by two washing steps in 1x PBS. The excess PBS was drained and the slides were incubated in 50 mL 1x PBS with 8µl proteinase K (concentration: 20µg/µl) for 8 min at

RT to make the cells accessible for the riboprobe. Tissue sections were incubated at 37°C to ensure an efficient digestion of proteins. This step was followed by a wash in 1x PBS for 5 min, refixation in 4% PFA for 5 min and three washing steps in 1x PBS, each 5 min. The sections were then acetylated in 50 mL 0.1M TEA-HCL including 125 µL acetic anhydride - followed by three washing steps in 1x PBS. Subsequently, the sections were dehydrated in 70% EtOH for 5 min, then in 95% EtOH for a few seconds and air-dried on paper towels. Approximately 1 µg of the desired riboprobe was added to 1 mL hybridization-buffer and heated up to 80°C for 2 min to denature the RNA. The following steps, in which pre-heated formamide was used were carried out under the fume hood. The slides were placed horizontally into the hybridization cassette of the hybridization oven, which was prepared with 50 mL of formamide and ddH20 (in a ratio of 1:1) and preheated in the hyboven at 55°C. 300 µL of the riboprobe/hybsol mix was added to each slide and covered with RNase-free hybridization coverslips. After that, the cassette was inserted in the hyboven and hybridized at 55°C o/n (12-18 h).

On the following day, the coverslips were removed in prewarmed 5x SSC and the slides were submitted to a high stringency wash in Formamide and 2x SSC (in a ratio of 1:1) for 30min at 65°C to reduce background by destabilizing less stable RNA-DNA duplexes. Then, three washing steps with RNase Buffer for 10 min at 37°C were performed followed by incubation in RNase Buffer + RNaseA (0.05 µg/ml) for 30 min at 37°C and one last RNase Buffer wash for 15 min at 37°C. After the removal of the non-hybridized RNA, the high stringency wash (see above) was repeated two times for 20 min at 65°C. Two additional washing steps in 2x SSC and 0.1x SSC for 15 min at 37°C were followed by a washing step in PBT (1x PBS with 0.1% Tween-20) for 15min at RT. A humidified box was prepared; the slides were placed horizontally in the box and blocked with 10% normal goat serum in PBT for 1 h. Next, the blocking solution was removed and the sections were incubated with Alkaline-phosphatase coupled Anti-DIG-AP Fab fragments diluted 1:5000 in PBT with 1% normal goat serum o/n at 4°C.

On the third day, the antibody was removed and the sections were washed four times in PBT at RT for 5-10min. Another two washes in freshly prepared alkaline phosphatase buffer (NTMT) containing 0.5mg/ml levamisole for 10 min at RT followed. Levamisole reduces background staining by inhibiting endogenous alkaline phosphatase activity. After that the sections were incubated in BM purple solution

(substrate for the alkaline phosphatase) containing 0.5mg/mL levamisole at RT. Depending on the riboprobes it took 4-24 h to detect a strong signal on the sections.

When the sections were adequately stained, a washing step in 1x PBS for 5min, a postfixation in 4%PFA for 5 min and two additional washing steps in 1x PBS and dH20 for 5 min followed, before the sections were mounted in AquaPolymount, a glycerol based mounting medium. Sections were stored in slide boxes at RT.

2.13 Enzyme linked immunosorbent assay (ELISA)

ELISA is an analytic biochemistry assay that detects the presence of an antigen in a liquid sample. The sample is given into the plate and the specific antigen binds the antibody of interest, which is then detected by a secondary antibody. For final detection, the appropriate chromogen substrate is given and this can be measured photometrically. During this study, various kits were used for this purpose (listed in table below). Thereby, the presence and concentration of different proteins was measured in peritoneal fluid and serum of women with and without endometriosis.

Protein	Company
Semaphorin 3C	USCN Business Co., Ltd., USA
Semaphorin 3F	USCN Business Co., Ltd., USA
Neuropilin 1	USCN Business Co., Ltd., USA
Neuropilin 2	USCN Business Co., Ltd., USA
DOP/NAD/ADR	Biozol Diagnostica GmbH, Germany
Interleukin-1β	BioLegend, USA
Interleukin-4	BioLegend, USA
Interleukin-10	BioLegend, USA
Interleukin-13	BioLegend, USA
TGF-β	Biozol Diagnostica GmbH, Germany

Proteins of interest were analyzed in the samples as shown in the following table:

		EM											Control						
		total	pelvic pain		cycle phase				rASRM				total	pelvic pain		cycle phase			
			yes	no	pro	sec	men	OC	I	II	III	IV		yes	no	pro	sec	men	OC
Peritoneal fluid	Sema 3C	13	10	5	4	3	0	0	3	5	1	1	19	9	10	4	7	0	0
	Sema 3F	14	10	4	4	3	0	0	2	4	4	0	14	7	2	4	6	0	0
	Nrp1	19	16	2	7	5	0	0	1	4	5	1	28	11	15	1	14	0	0
	Nrp2	13	11	2	5	2	0	5	2	5	2	2	13	7	4	2	3	0	5
	DOP	17	13	1	2	4	1	7	6	3	2	4	6	4	0	0	0	0	1
	NAD	26	22	2	3	7	5	9	9	6	2	8	9	6	0	0	2	0	1
	ADR	18	16	1	2	5	1	7	5	4	2	4	13	5	0	0	1	0	1
	IL-1ß	11	9	0	3	4	0	0	3	5	0	1	10	5	2	0	5	1	0
	IL-4	22	17	1	7	5	0	0	4	1	6	5	17	12	2	5	4	0	0
	IL-10	21	16	3	8	5	0	0	4	2	3	5	18	8	2	5	5	0	0
	IL-13	32	28	0	8	12	0	0	7	11	5	4	32	18	5	10	12	0	0
	TGF-β	13	11	1	2	4	1	5	3	4	3	3	15	7	3	3	4	1	2
Serum	Sema 3C	15	12	2	2	3	0	0	4	3	2	5	6	2	6	0	3	0	0
	Sema 3F	16	13	1	1	3	0	4	4	1	2	7	8	2	5	1	2	0	1
	Nrp1	10	8	1	1	3	0	3	2	1	0	4	8	2	5	0	4	0	1
	Nrp2	16	14	1	3	3	0	4	4	3	2	5	9	4	5	1	4	0	1
	DOP	16	14	0	2	3	0	5	4	3	2	5	8	2	5	0	2	1	3
	NAD	7	7	0	2	3	0	0	2	1	2	2	6	2	2	0	2	0	0
	ADR	14	11	0	1	3	0	0	3	2	1	5	3	1	2	0	2	0	1
	IL-1ß	6	5	1	2	1	0	0	3	1	1	0	2	0	2	1	1	0	0
	IL-4	9	8	1	1	3	0	0	2	0	1	4	7	3	4	1	2	0	0
	IL-10	9	8	1	1	3	0	0	2	1	0	3	7	3	4	1	2	0	0

2.14 Cell culture

Cell line:

The cell line used for experiments in this work was L929. It is a fibroblasts-like cell line cloned from strain L, which was established by WR Earle in 1940. The parent strain was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse.

Medium:	RPMI 160	500 ml (88%)
	FCS	57 ml (10%)
	2 mM glutamine	5.7 ml (1%)
	Pen/Strep	5.7 ml (1%)

Cell thawing:

Medium was prewarmed at 37°C. Cells were thawed in waterbath at 37°C (not fully). Subsequently, cells were transferred into a falcon tube and 13 ml PBS were added and immediately centrifuged for 10 minutes at 1100 rpm. Supernatant was removed and 5 ml of the prewarmed medium was added to the cells, which was then transferred into a 25 cm^2 culture flask. On the next day, cells were checked and if necessary passaged.

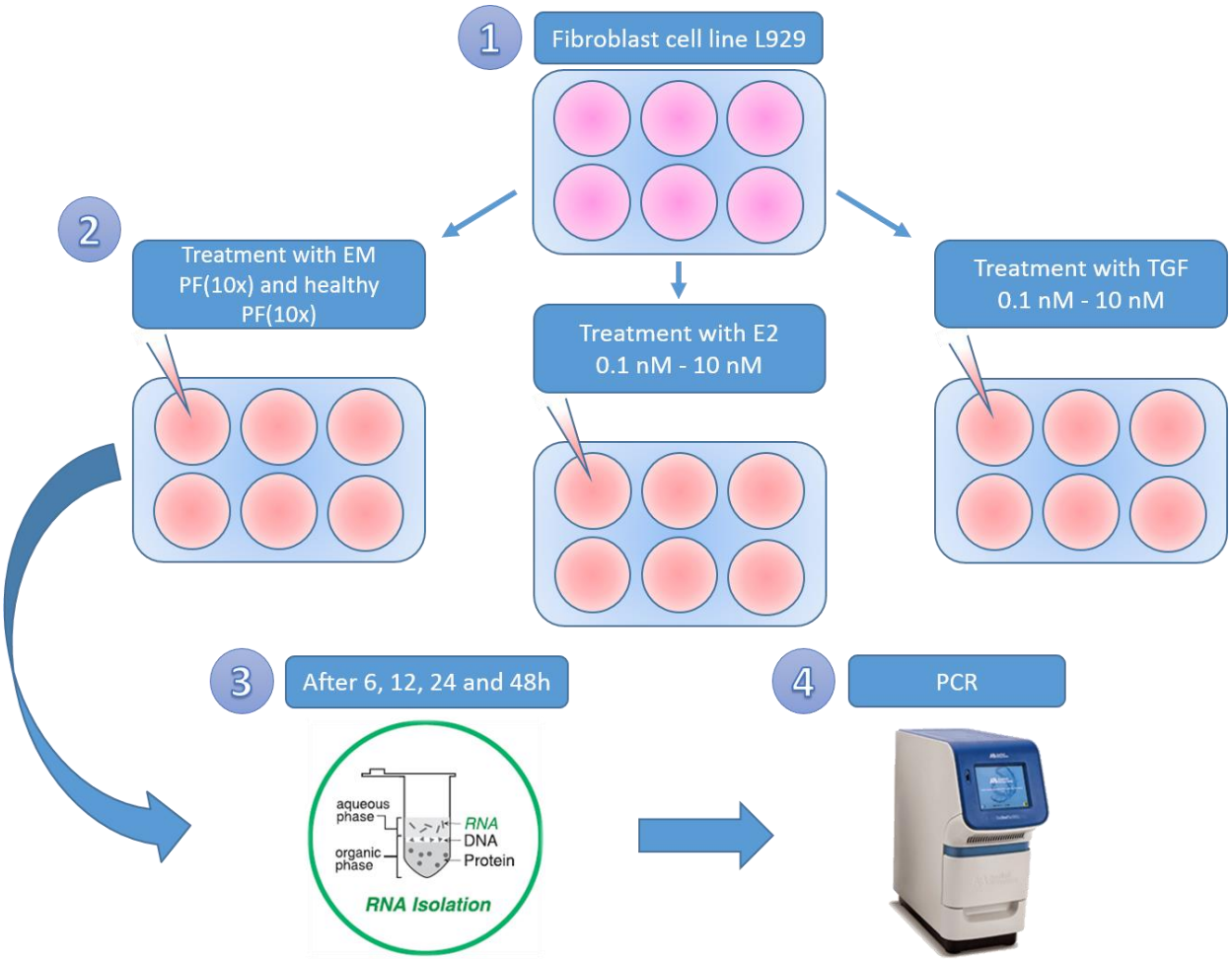
Cell passaging/splitting:

Required volume of medium was prewarmed at 37°C. The cell medium in the flask was removed and cells were washed two times with 5 ml PBS. Consequently, 1 ml trypsin (for 25 cm^2 flask or 2 ml trypsin for 75 cm^2 flask) was added to loose the cells from the flask. Trypsin is kept on cells until cells are detached. Side of flask was taped on the hand to help cells detach. Under the microscope, the detachment of the cells was verified. When cells were detached, 5 ml of the prewarmed medium (for 25 cm^2 flask or 10 ml medium for 75 cm^2 flask) was added. Then, cells were splittet (1:4). Small flasks were filled up to 10 ml medium, big flasks were filled up to 20 ml medium. Passaging of the cells was done every 3-4 days.

Cell treatment experiments:

In this study, L929 cells were treated with peritoneal fluid of 10 women with endometriosis and 10 women without endometriosis. Furthermore with estrogen (0.1 nM-10 nM) and TGF (0.1 nM-10 nM). The treatment was stopped after 6, 12, 24 and 48 hours by extraction of RNA. Every probe had a duplicate which was treated identically.

For experimental set-up see following picture:

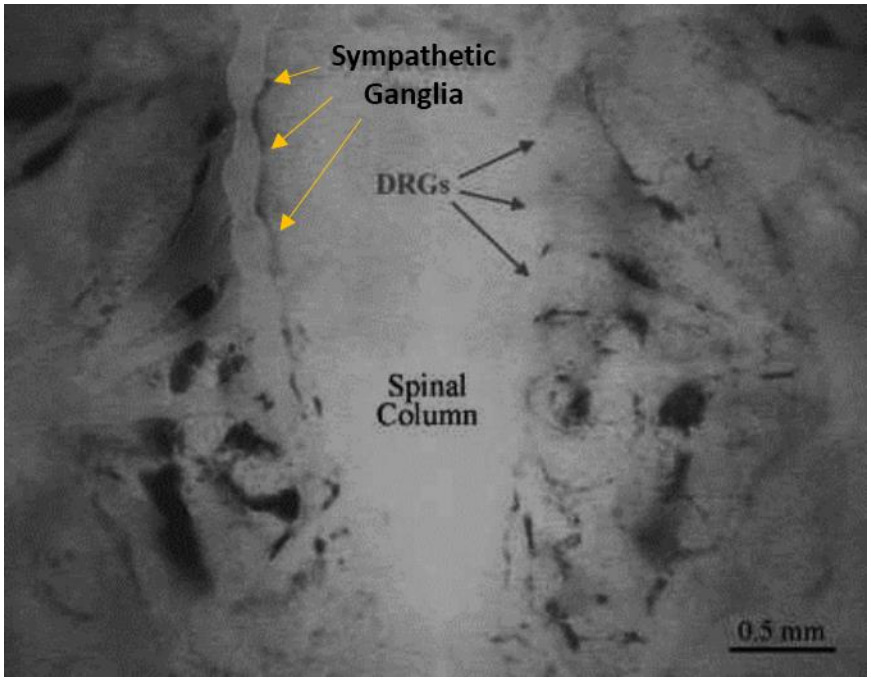


2.15 Neuronal outgrowth assay

Embryonic sympathetic ganglia from 8-day-old chicken were prepared for experiments. With this method, the neurite outgrowth of sympathetic ganglia are measured. The fertilized eggs were incubated in an automated incubator with a motorized egg turner with a humidity of 60% at 37°C for 8 days.

For the ganglia preparation, the eggs were disinfected with ethanol, and then opened at the round side (air cell) with a large tweezer. Consequently, the embryos were taken out of the egg and immediately decapitated. After decapitation, the embryo body was put in a petri dish with cold PBS with the ventral side up. Organs in chest and abdomen were removed. Under a binocular, connective tissues surrounding the spinal column were removed carefully, getting access to the sympathetic chain, which was then

carefully taken out with a small and thin tweezer. The ganglia were collected in a fresh petri dish with PBS.



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Well plates were prepared one day before dissection. The wells were treated with collagen IV (1:50 diluted with PBS) and incubated over night at 4°C. On the dissection day, collagen IV solution was removed and the wells were washed three times with PBS. Consequently, wells were filled with Dulbecco´s modified eagle medium (DMEM) and the ganglia were put in the wells.

For the experiments, ganglia were first incubated for 24 hours with 0.1 ng/ml NGF to promote outgrowth (positive control) or without NFG as a negative control. The incubator had always 37°C and 5% CO2. After 24 hours incubation with NGF, ganglia were treated with peritoneal fluid, semaphorins, or both; again for 24 hours. Every sample had a duplicate. The neurite outgrowth was evaluated under the microscope.

2.16 Software

Image editing	
Illustrator CS5	Adobe Systems Incorporated
ImageJ 1.43u	National Institutes of Health (USA)
Microsoft Picture Manager	Microsoft Office (California, USA)
Photoshop CS5	Adobe Systems Incorporated

Imaging	
AxioVision Real 4.7	Zeiss (Jena, Germany)
Leica Application Suite 3.3.0	Leica (Wetzlar, Germany)
Remote Capture	Canon GmbH (Germany)
Zen 2009	Carl Zeiss AG (Germany)

Word processor	
Microsoft Office 2010	Microsoft Germany
EndNote X7	Thomson Reuters (USA)

3 Results

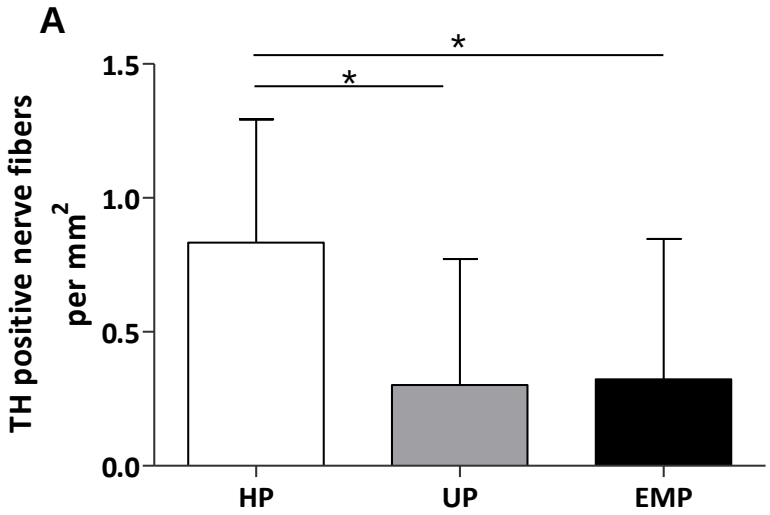
3.1 Tissue analysis

3.1.1 Quantification of noradrenergic nerve fibers in peritoneal endometriosis

The appearance of TH (tyrosine-hydroxylase) positive nerve fibers was quantified in peritoneal biopsies of women with and without endometriosis (healthy peritoneum). Further, the peritoneal tissue of women with endometriosis was subgrouped in peritoneum with macroscopically visible lesions (affected peritoneum) and peritoneum with macroscopically no visible lesions (unaffected peritoneum).

Noradrenergic nerve fibers appearance is lower in affected peritoneum and unaffected peritoneum in comparison to healthy peritoneum (mean TH-positive nerve fibers/mm2: 0.32±0.09; 0.30±0.10 and 0.83±0.19 respectively) (Fig. 1 A-G).

Noradrenergic nerve fibers could be identified accompanied by vessels in the affected and unaffected peritoneum of women with endometriosis. In the peritoneal tissue of women without endometriosis, noradrenergic nerve fibers were not always accompanied by vessels, but rather appeared alone.



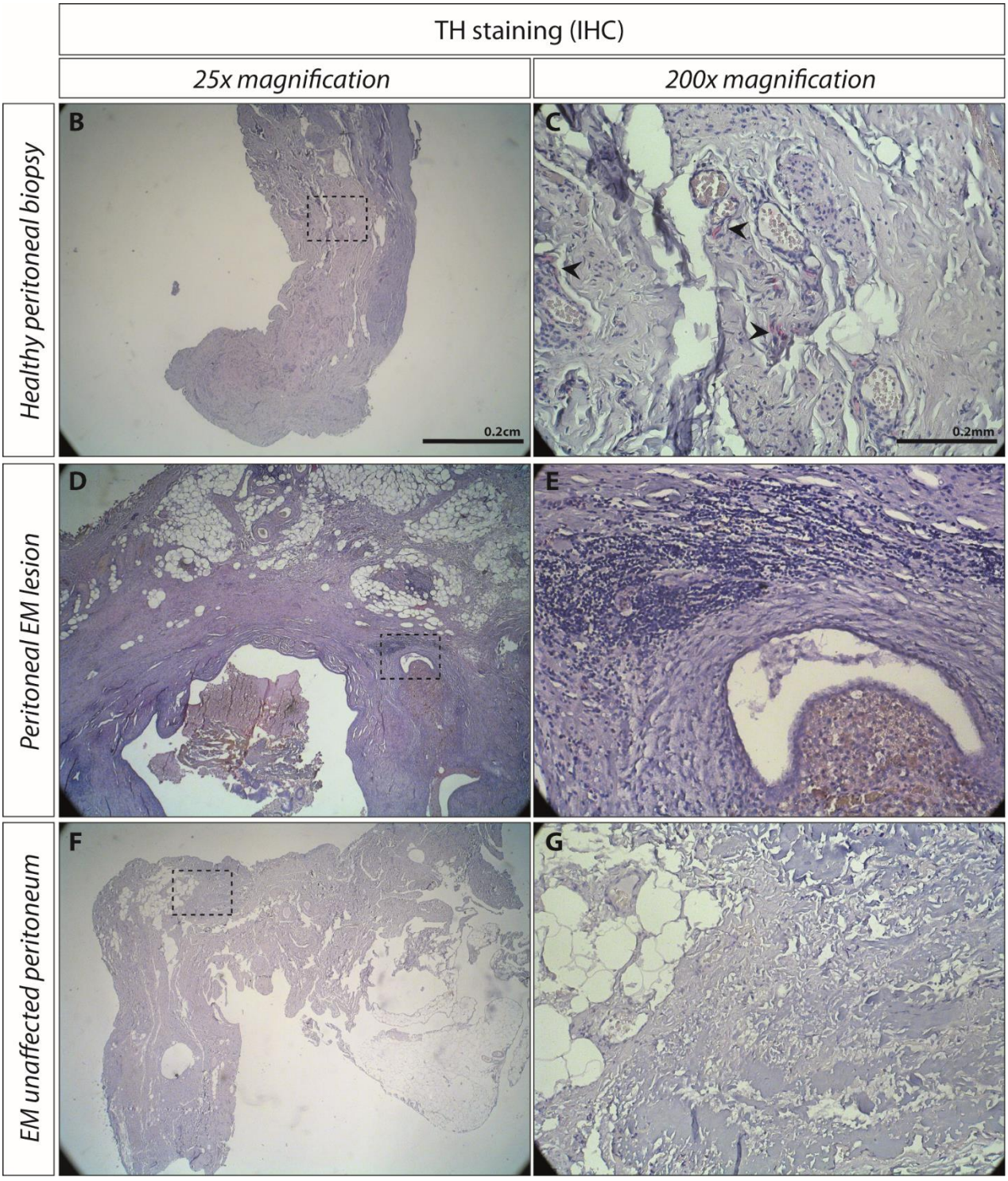


Figure 1: Quantification of noradrenergic nerve fibers in peritoneum of women with and without endometriosis (A). Noradrenergic nerve fibers in healthy peritoneal biopsy (B-C), peritoneal endometriosis lesion and endometriosis unaffected peritoneum (F-G). HP: healthy peritoneum, UP: endometriosis unaffected peritoneum, EMP: affected peritoneum. *p< 0.05

3.1.2 Sema3C and 3F are expressed in endometriotic peritoneum

The expression of Sema3C and 3F was analyzed through RNA in situ hybridization in peritoneal endometriosis. The analysis revealed the expression of Sema3C and 3F at RNA level in peritoneal endometriotic tissue (Fig. 2 A-B).

The expression of the named Semas was verified by immunohistochemical analysis in the same tissue, revealing Sema3C and 3F positive cells close to the endometriotic lesion (Fig. 2 C). By morphological analysis of the histological stainings, the Sema positive cells were identified as endothelial cells of blood vessels, stromal like cells, and a variety of immune cells. In the unaffected tissue of women with endometriosis and healthy peritoneum, Sema 3C and 3F were only expressed in scattered cells in the connective tissue, presumably fibroblasts (Fig. D-E and G-H).

The statistical analysis revealed an overall significantly higher occurrence of Sema3C positive cells in endometriotic tissue, when compared to unaffected or healthy peritoneum (mean cells/mm2: 27.76±2.64, 2.92±1.09 and 2.33±1.01, respectively) (Fig. I). Similarly, Sema3F expression is significantly increased in comparison to unaffected or healthy peritoneum (mean cells/mm2: 23.61±2.50, 4.08±1.19 and 3.56±2.48, respectively) (Fig. 1J).

Analysis of Semas expression in dependence of EM stage or menstrual cycle phase revealed no significant difference. Equally, the analysis of Sema expression in tissue of women with endometriosis, experiencing pelvic pain and without painful symptoms, yielded no significant differences (Sema 3C pain vs. no pain: mean cells/mm²: 26.03±2.99 and 32.57±5.74; Sema 3F pain vs. no pain: mean cells/mm²: 23.10±2.88 and 24.17±6.75).

Semas expression could be found in increased amounts near endometriosis lesions. However, until now only morphological studies could elucidate a more detailed characterization of the Sema positive cells. Recent studies have demonstrated, specific immune cells as important Semas producers. Therefore, in this study the expression of Sema3C and Sema3F was analyzed in specific immune cells, such as macrophages and activated fibroblasts.

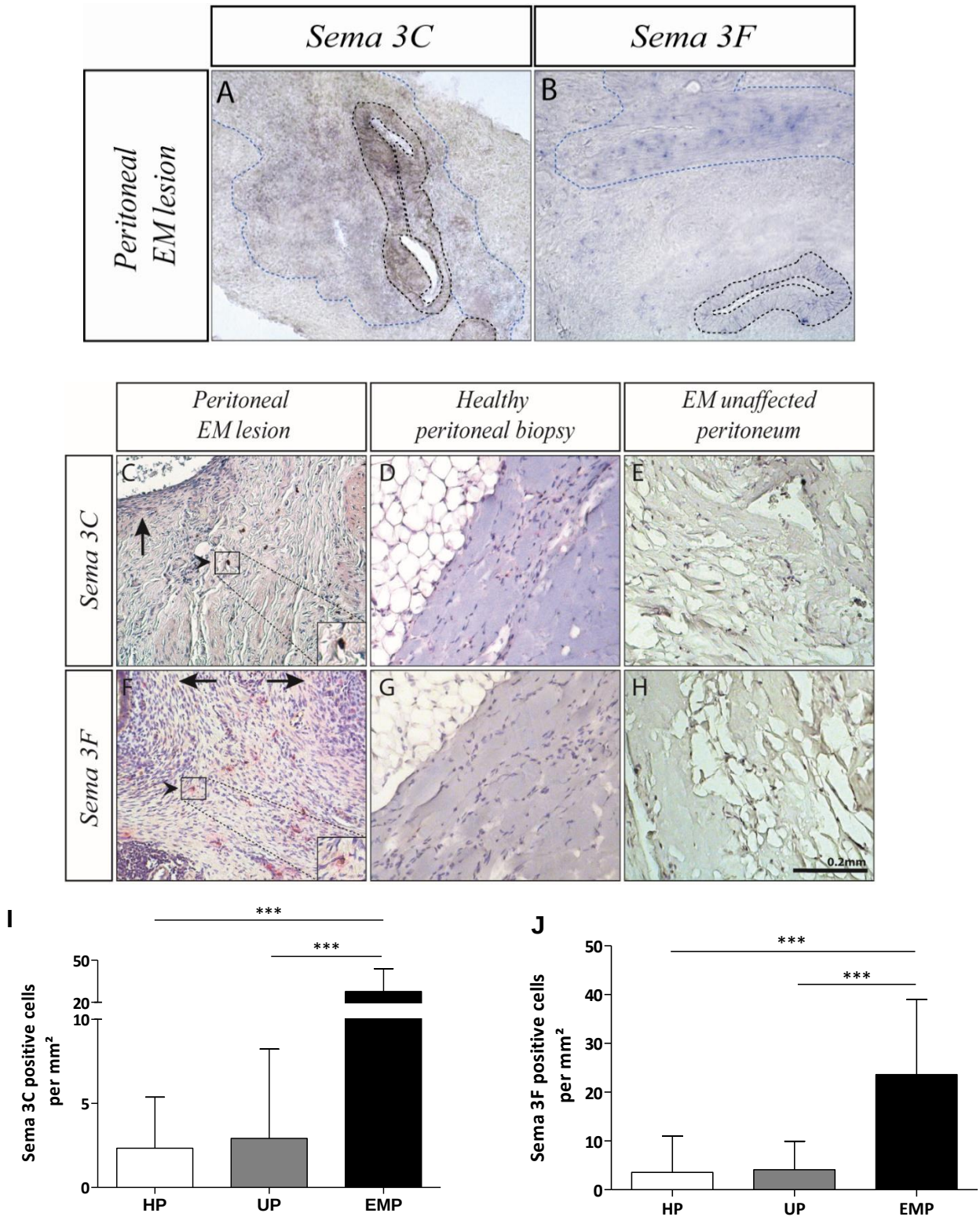


Figure 2: Sema 3C and 3F expression at RNA level in peritoneal endometriotic tissue (A-B). Sema 3C and 3F expression in Peritoneal endometriosis (A and D), healthy peritoneal biopsy (B and E) and EM unaffected peritoneum (C and F). Statistical analysis of Sema 3C and 3F expression in healthy peritoneum of women without endometriosis (HP), unaffected peritoneum of women with endometriosis (UP) and endometriotic peritoneum (EMP) (G and H). ***p< 0.001.

3.1.3 Higher macrophage occurrence in endometriosis and Sema secretion

The amount of peritoneal macrophages in the affected peritoneum of women with endometriosis is significantly higher than in unaffected peritoneum or healthy peritoneum (mean macrophages/mm2: 7.46±0.90, 0.50±0.13 and 0.22±0.17, respectively; Kruskal Wallis test with Dunn´s comparison: $p < 0.001$) (Fig. 3 A-G). Analysis of macrophages appearance in dependence of EM stage or menstrual cycle phase revealed no significant difference.

Furthermore, CD68 positive macrophages are Sema3C positive, the double staining revealed that 86.62% of macrophages in endometriotic peritoneum also express Sema3C, while only 50% of macrophages found in unaffected peritoneum express Sema3C. In the healthy peritoneum, no macrophages could be identified. Similarly, Sema 3F was expressed in 96.64% of the peritoneal CD68 positive macrophages in the endometriosis group whereas in the unaffected peritoneum of women with endometriosis only 33.33% of the macrophages were Sema 3F positive. In the control, very few macrophages could be identified, and these were to 75% Sema 3F positive.

Probably, many of the macrophages identified in the endometriotic peritoneum express both Sema3C and Sema3F. The lower amount of macrophages in unaffected peritoneum and healthy peritoneum might be the cause of no elevated appearance of Semas in this tissue.

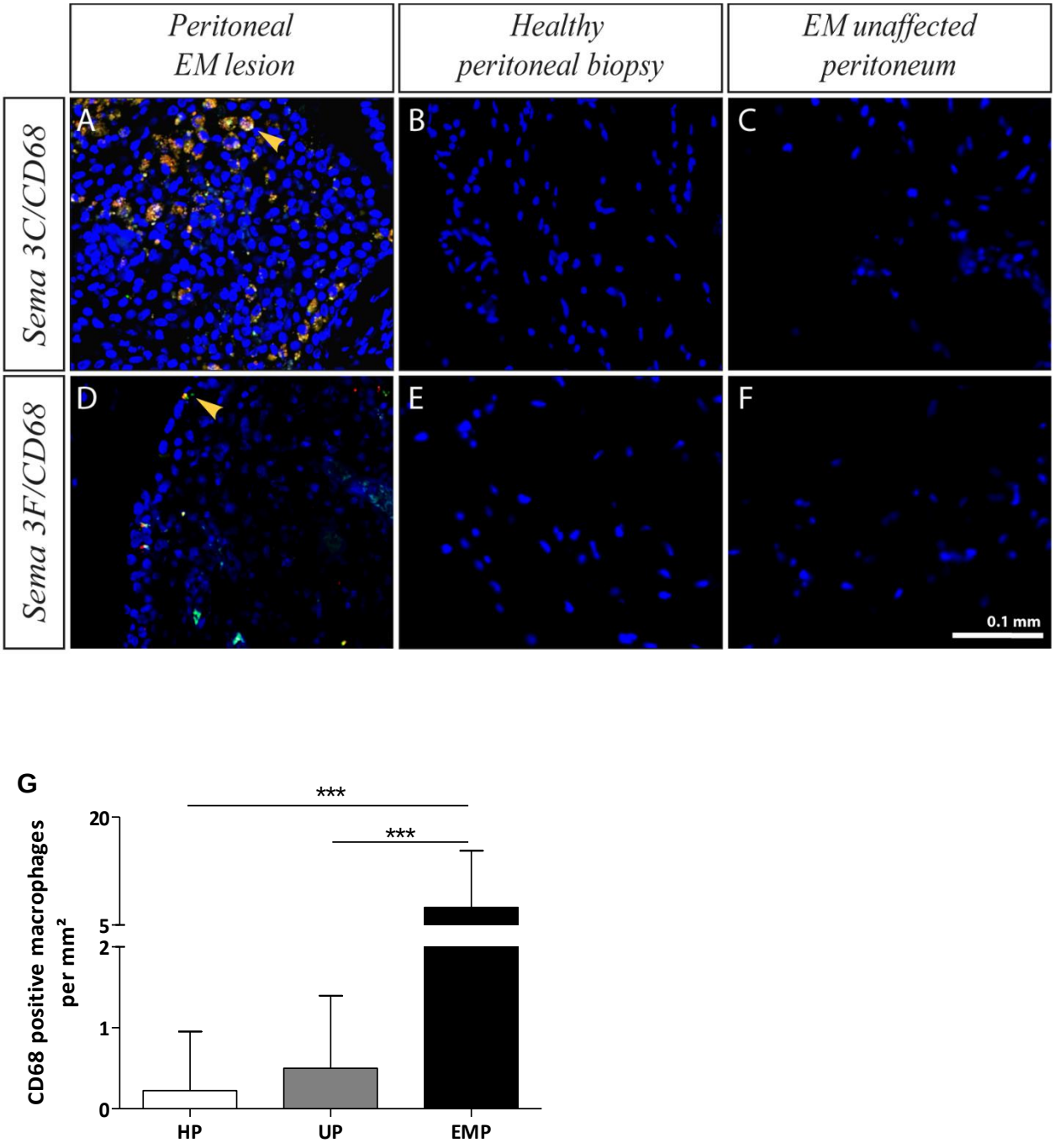


Figure 3: Immunofluorescence double staining of Sema 3C/3F and CD68 in peritoneal endometriotic tissue (A and D), healthy peritoneal biopsy (B and E) and endometriosis unaffected peritoneal biopsy (C and E). Arrowheads show colocalization. (G) Quantification of the amount of macrophages in endometriotic peritoneum (EMP), in unaffected peritoneum of women with endometriosis (UP) and in healthy peritoneum (HP) of women without endometriosis (mean and standard deviation; Kruskal Wallis test with Dunn's multiple comparison test). ***p< 0.001.

3.1.4 Activated fibroblasts in peritoneal endometriosis express Sema3C and Sema3F

Activated fibroblasts were mainly identified in tissue of women with endometriosis. Here, the identified fibroblasts were positive for Sema3C and Sema3F. Nevertheless, the amount of activated fibroblasts found in the unaffected peritoneum of women with endometriosis was definitively extremely lower than in the affected peritoneum; in the unaffected peritoneum only few scattered activated fibroblasts could be identified. Similarly, in the healthy peritoneum only scattered activated fibroblasts were found; these were not Sema positive (Fig. 4).

Activated fibroblasts were identified between stromal cells, surrounding endometriosis lesions and in the fibro connective tissue, which surrounds the fat tissue of the peritoneum of women with endometriosis.

The low amounts of activated fibroblasts, as well as macrophages might also contribute to the low appearance of Semas expression in unaffected and healthy peritoneum. For Semas signal transduction, the presence of specific receptors is required. Therefore, the expression of these receptors was analyzed on the tissue samples.

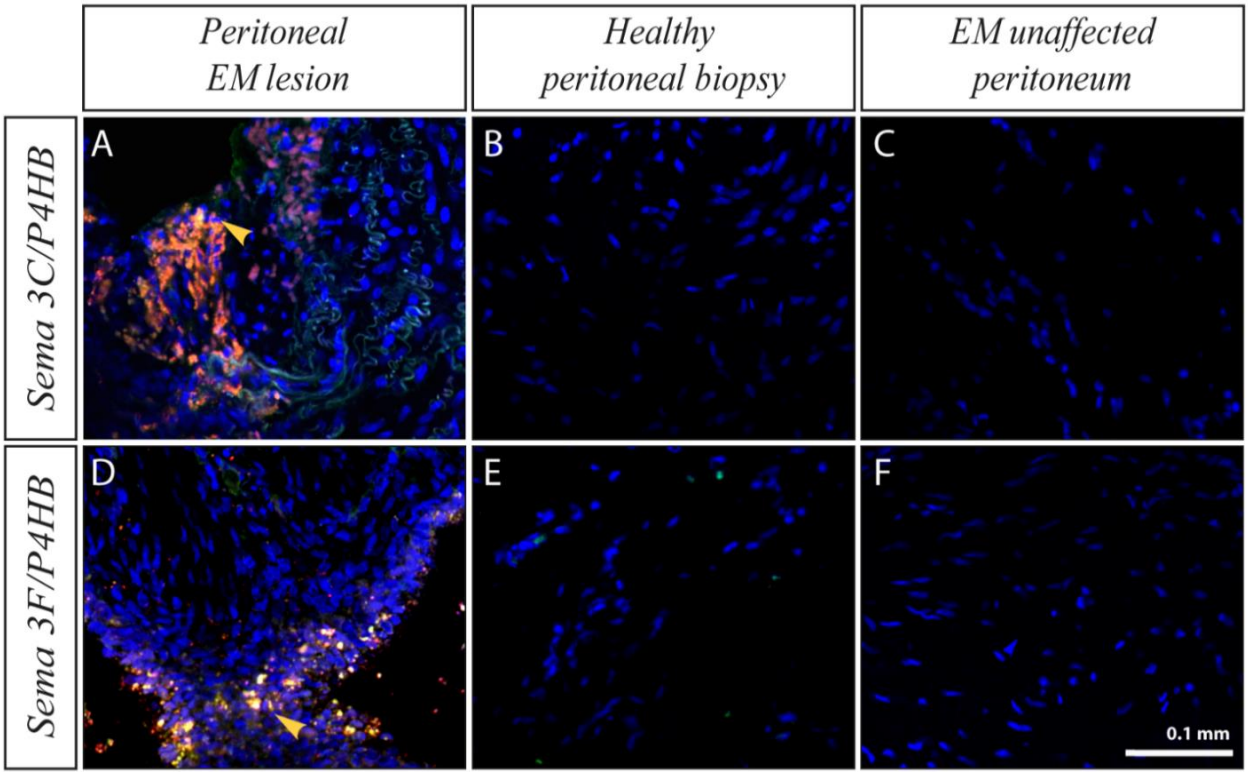


Figure 4: Immunfluorescence double staining of Sema3C/3F and P4HB. Sema 3C/3F with P4HB staining in peritoneal endometriotic tissue (A and D), healthy peritoneum of women without endometriosis (B and E), and unaffected peritoneum of women with endometriosis (C and F). Arrowheads sema expression in P4HB-activated fibroblasts.

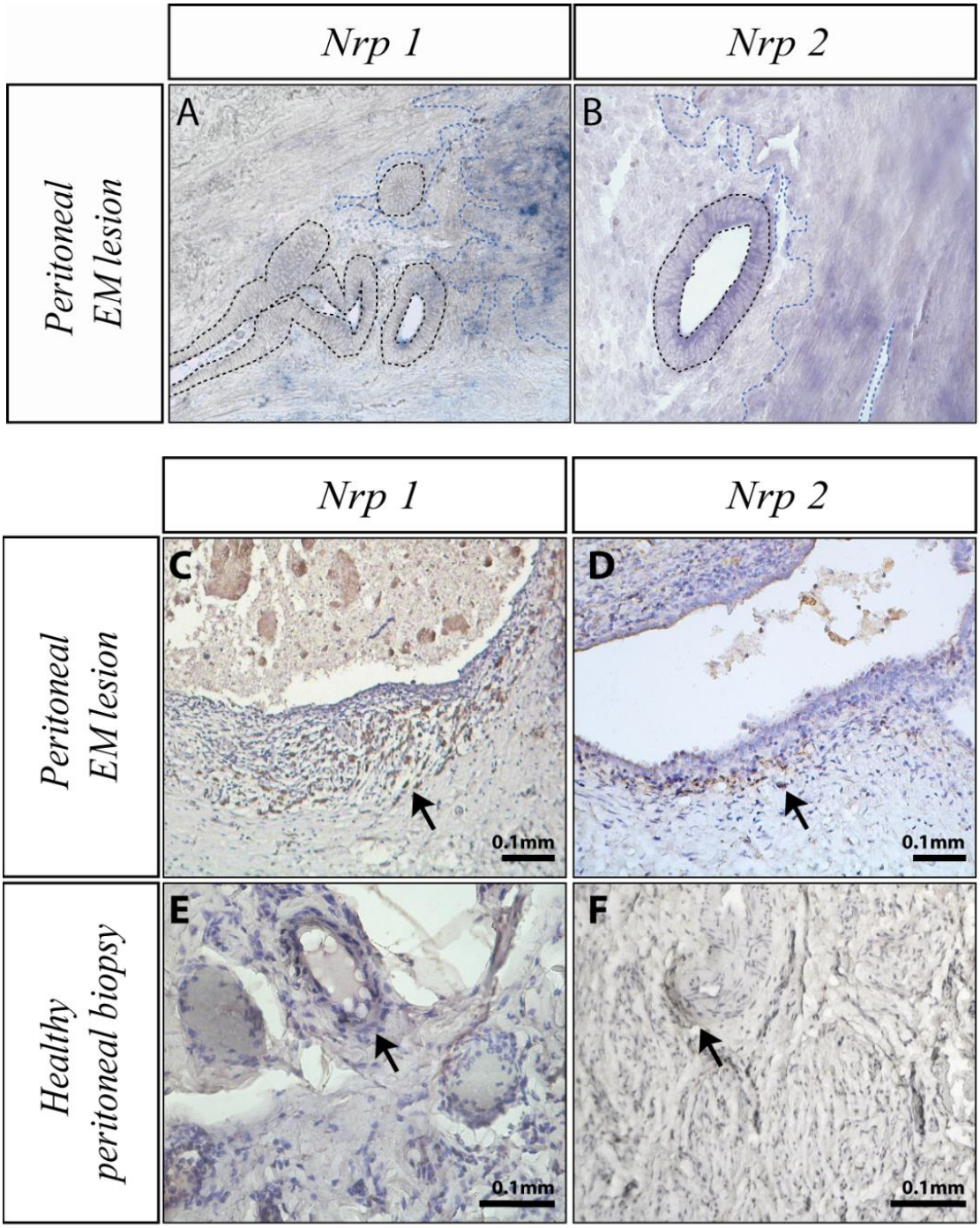
3.1.5 Peritoneal endometriotic tissue is Nrp1 and Nrp2 positive

Nrp1 and Nrp2 are the main receptors of the analyzed Semas. Expression of both receptors could be identified via RNA in situ hybridization in affected tissue of women with endometriosis in the surroundings of the lesion and the lesion (Fig. 5 A-B).

The expression of both receptors was verified at protein level through immunohistochemical staining. This analysis revealed the expression of Nrp1 and Nrp2 in the affected peritoneum of women with endometriosis (Fig. 5 C-D).

In peritoneal tissue of women without endometriosis, Nrp1 and Nrp2 expression could only be identified in blood vessels (Fig. 5 E-F and I-J). Through morphological analysis, the expression of the receptors was attributed to stromal cells, immune cells, endothelial cells and blood vessels in the direct surroundings of an endometriosis lesion. Nevertheless, the surrounding fibro connective tissue also showed, even though to a lesser extent, Nrp1 and Nrp2 expression.

Sema induced nerve repellence requires expression of the specific receptors on the membrane of the nerve fibers supposed to be repelled. Therefore, it was important to determine whether the nerve fibers found in endometriosis are positive for Nrp1 and Nrp2.



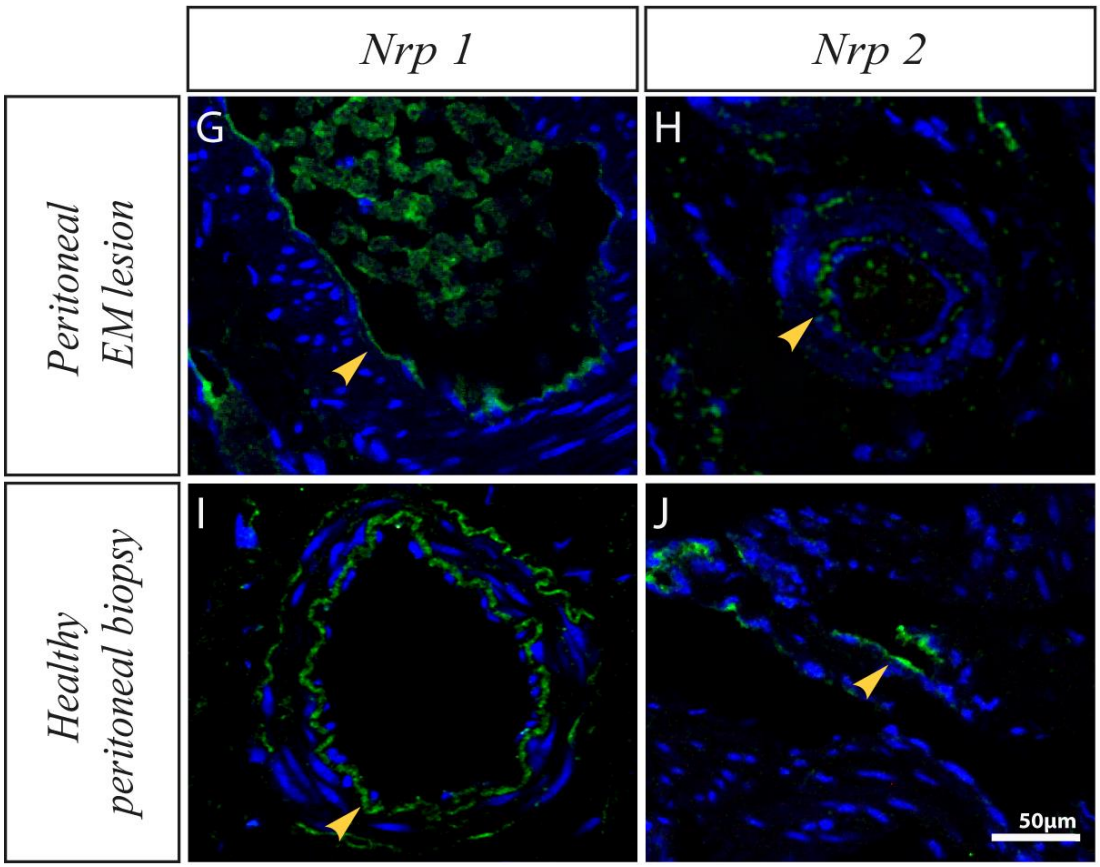


Figure 5: Expression of Nrp 1 and Nrp 2 in peritoneal endometriotic lesions. RNA in situ hybridization of Nrp 1 and Nrp 2 in peritoneal endometriotic tissue (A-B). Endometriosis lesion is marked with the black dashed line and RNA positive regions are marked with the blue dashed line. (C-F) Immunhistochemical staining of Nrp 1 and Nrp 2 in peritoneal endometriotic lesions and healthy peritoneum of women without endometriosis respectively. Arrows point at Nrp positive cells. (G-J) Immunfluorescence staining of Nrp 1 and Nrp 2- positive blood vessels in peritoneal endometriotic tissue and healthy peritoneum of women without endometriosis. Arrowheads show Nrp 1 or Nrp 2 expression (green).

3.1.6 Endometriosis associated noradrenergic nerve fibers express Nrp1 and Nrp2

Noradrenergic nerve fibers in the peritoneal samples of women without endometriosis, were as expected always negative for Nrp1 and Nrp2. In contrast, noradrenergic nerve fibers identified in peritoneal samples of women with endometriosis, independent of the appearance of a lesion, showed Nrp1 and Nrp2 expression (Fig. 6 A-F).

Interestingly, tyrosine hydroxylase staining seem to be stronger in samples of women without endometriosis than in peritoneum of women with endometriosis (Fig. 6 A-B and D-E).

Further receptors are important for Sema induced nerve fiber repellence. Therefore, PlexinA3 and A4 were also analyzed in endometriosis associated noradrenergic nerve fibers.

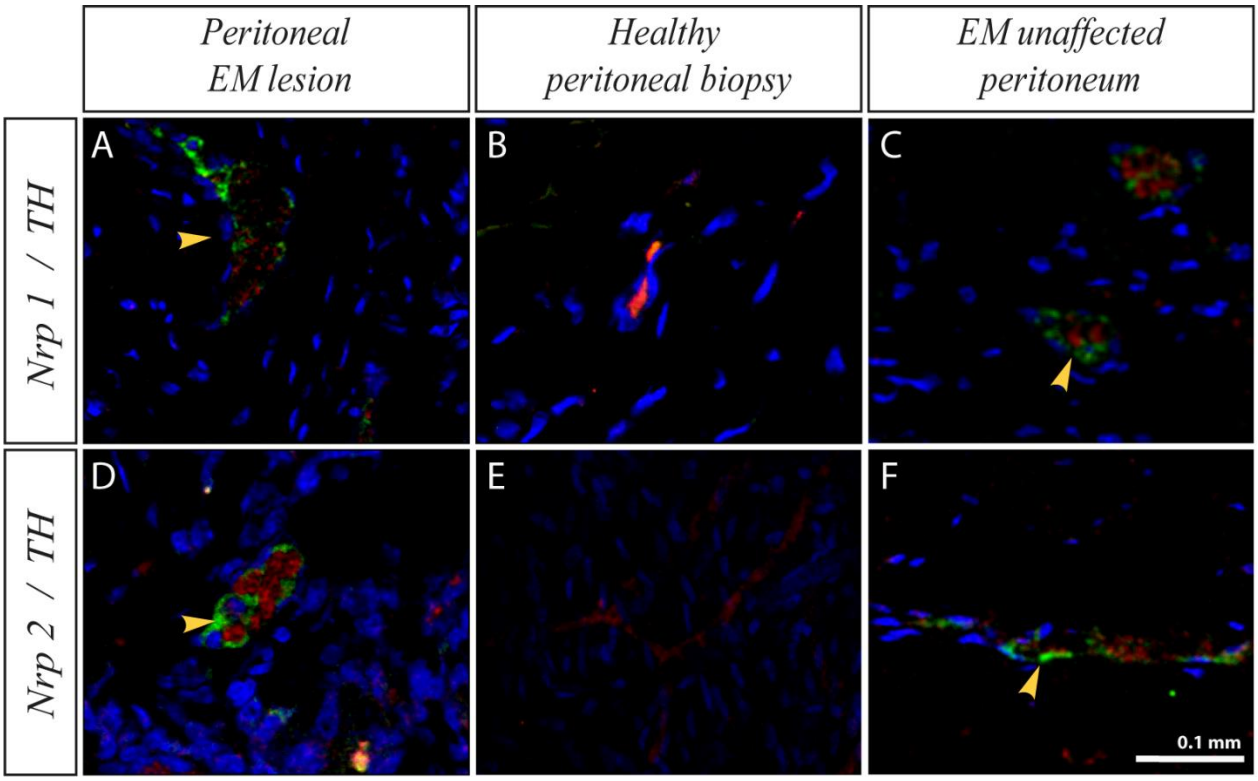


Figure 6: Immunfluorescence double staining of neuropilins and tyrosine hydroxylase. NRP1/TH staining in peritoneal endometriotic tissue (A), healthy peritoneum of women without endometriosis (B), and unaffected peritoneum of women with endometriosis (C). NRP2/TH staining in peritoneal endometriotic tissue (D), healthy peritoneum of women without endometriosis (E), and unaffected peritoneum of women with endometriosis (F). Nrp appears green, TH appears red. Arrowheads show Nrp expression on membrane of TH-positive nerves.

3.1.7 Endometriosis associated noradrenergic nerve fibers express Plexins

Noradrenergic nerve fibers in the peritoneal samples of women with endometriosis (affected and unaffected) show PlexinA3 and A4 expression. Conversely, noradrenergic nerve fibers in peritoneal samples of women without endometriosis were negative for both Plexins (Fig.7 A-F).

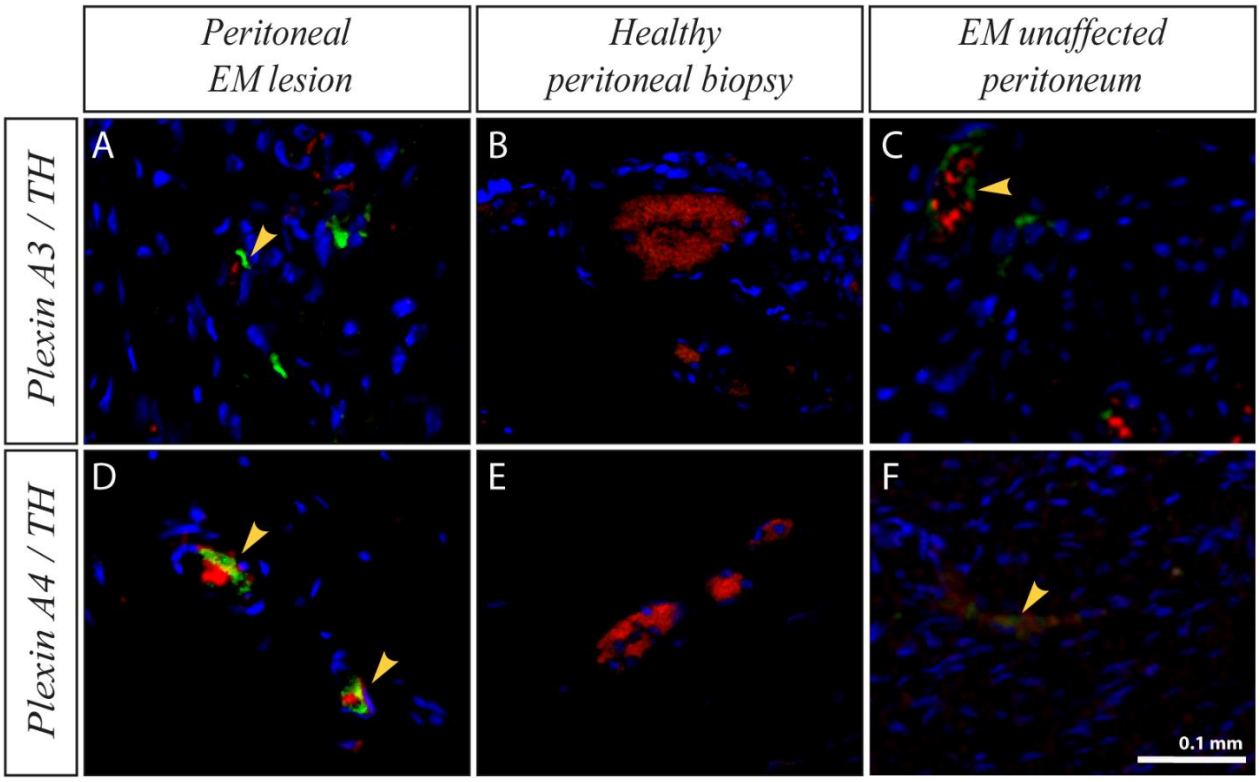


Figure 7: Immunfluorescence double staining of plexins and tyrosine hydroxylase. Plexin A3/TH staining in peritoneal endometriotic tissue (A), healthy peritoneum of women without endometriosis (B), and unaffected peritoneum of women with endometriosis (C). Plexin A4/TH staining in peritoneal endometriotic tissue (D), healthy peritoneum of women without endometriosis (E), and unaffected peritoneum of women with endometriosis (F). Plexin appears green, TH appears red. Arrowheads show Plexin expression on membrane of TH-positive nerves.

3.2 Peritoneal fluid analysis

3.2.1 Peritoneal fluid of endometriosis patients has an elevated Sema3C but not Sema3F concentration

Expression of Sema3C and 3F was analyzed in peritoneal fluid through ELISA analysis. This revealed an increased concentration of Sema3C in peritoneal fluid of EM patients when compared to peritoneal fluid of women without endometriosis (mean Sema3C ng/ml: 23.56±3.22 and 17.73±4.02 respectively. Mann-Whitney test. p = 0.0432) (Fig.8).

No significant difference in Sema3C expression in dependence of endometriosis stage (mean Sema3C ng/ml: rASRMI 28.71±10.86, rASRMII 20.90±2.41, rASRMIII 9.80±ND, rASRMIV 18.15±ND respectively. Kruskal-Wallis test p = 0.47. ND: no data), cycle phase (mean Sema3C ng/ml: 26.90±4.95, 23.98±9.02 in the secretory and proliferative

cycle phase respectively; Mann-Whitney test. p = 0.4) or occurrence of pelvic pain (mean Sema3C ng/ml: EM with pelvic pain 24.31±2.90, EM no pelvic pain 21.33±7.46 respectively. Mann-Whitney test. p = 0.25), could be identified.

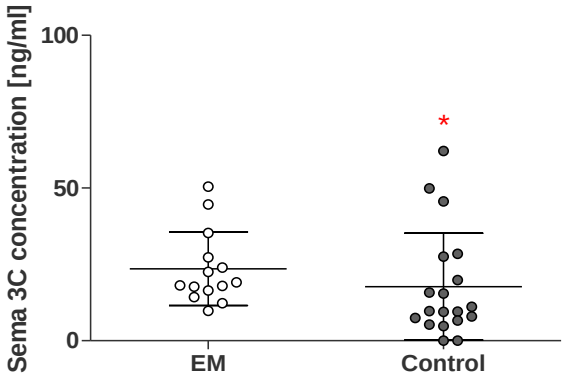


Figure 8: Sema 3C concentration in peritoneal fluid of women with endometriosis (EM) and without endometriosis (Control).*p<0.05

Sema3F expression is unaltered in peritoneal fluid of endometriosis patients (mean Sema3F ng/ml: 137.7±26.02 and 201.7±24.82 in EM and healthy PF respectively. Mann-Whitney test. p = 0.09) (Fig.9). Thereby, Sema3F expression seems to be independent of endometriosis stage (mean Sema3F ng/ml: rASRMI 281.21±18.79, rASRMII 158.16±12.45, rASRMIII 111.58±64.76, rASRMIV ND respectively. Kruskal-Wallis test p = 0.47. ND: no data), cycle phase (mean Sema3F ng/ml: 170.46±49.66, 131.29±20.50 in the proliferative and secretory cycle phase respectively; Mann-Whitney test. p = 0.18) or occurrence of pelvic pain (mean Sema3F ng/ml: EM with pelvic pain 161.6±30.54, EM no pelvic pain 77.78±39.96 respectively. Mann-Whitney test. p = 0.70), could be identified.

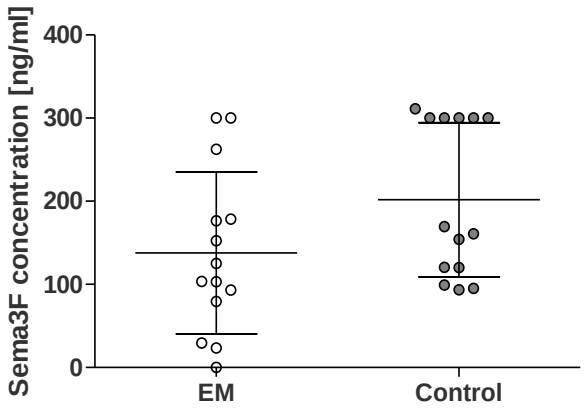


Figure 9: Sema 3F concentration in peritoneal fluid of endometriosis patients (EM) and women without endometriosis (Control).

Sema receptors, Nrp1 and Nrp2, exist in a soluble form and contribute to Sema action, so these were analyzed in peritoneal fluid.

3.2.2 Nrp1 expression is unmodified in peritoneal fluid of women with endometriosis

Nrp1 expression in peritoneal fluid of women with endometriosis is similar to the expression in peritoneal fluid of women without endometriosis. (±0.23 and 0.67±0.16 in endometriosis and healthy probes respectively. Mann-Whitney test. p = 0.84) (Fig. 10).

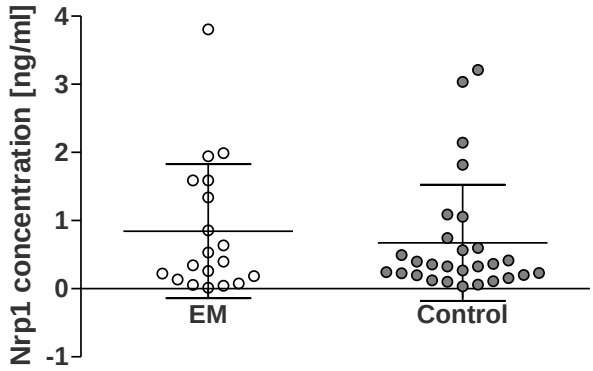


Figure 10: Nrp 1 concentration in peritoneal fluid of endometriosis patients (EM) and women without endometriosis (Control).

3.2.3 Nrp2 expression is increased in peritoneal fluid of endometriosis patients

Soluble Nrp2 expression could be identified in peritoneal fluid. When comparing the concentration of Nrp2 in peritoneal fluid of women with or without endometriosis, a significant higher concentration was revealed in the probes of diseased women (mean Nrp2 ng/ml: 206.3±54.60 and 80.12±25.25 respectively. Mann-Whitney test. p = 0.04) (Fig. 11 A).

Thereby, Nrp2 expression might be slightly dependent of the endometriosis stage, with higher concentrations in rASRM stage III (mean Nrp2 ng/ml: rASRMI 122.8±81.24, rASRMII 133.2±59.80, rASRMIII 594.9±5.13, rASRMIV 22.96±22.96 respectively. Kruskal-Wallis test p = 0.11). The menstrual cycle phase or intake of oral contraceptive did not significantly affect the Nrp2 concentration in peritoneal fluid of women with endometriosis (mean Nrp2 ng/ml: 245.3±95.85, 305.5±284.3 and 159.8±52.85 in the proliferative-, secretory cycle phase and under hormonal treatment respectively Kruskal-Wallis test p = 0.85). The occurrence of pelvic pain might be positively correlated with higher concentrations of Nrp2 in peritoneal fluid (mean Nrp2 ng/ml: EM with pelvic pain 234±9, EM no pelvic pain 49.16±7.60 respectively. Statistical test not possible due to limited sample number) (Fig. 11 B).

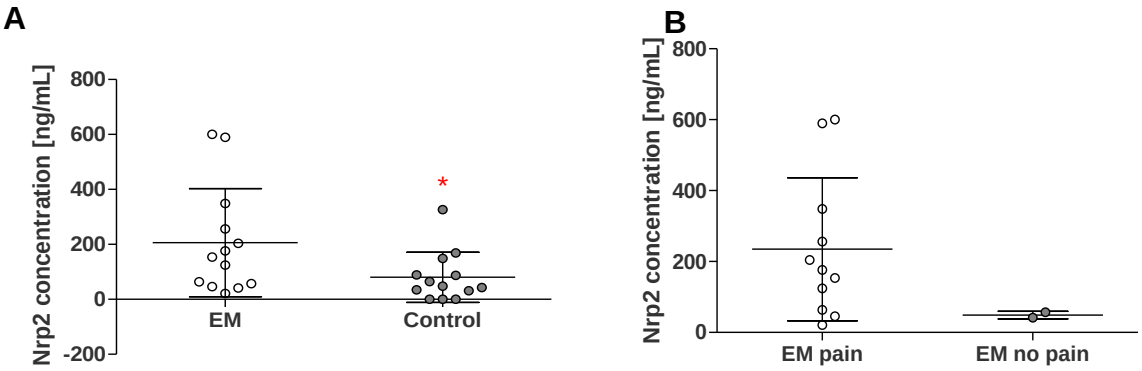


Figure 11: (A) Nrp 2 concentration in peritoneal fluid of endometriosis patients (EM) and women without endometriosis (Control). (B) Nrp 2 concentration in peritoneal fluid of endometriosis patients suffering of pelvic pain (EM pain) and endometriosis patients without pelvic pain (EM no pain).*p<0.05

These results demonstrate a negative correlation between appearance of noradrenergic nerve fibers and Semas/receptors expression in tissue and fluid. The reduced amount of noradrenergic nerve fibers might lead to a decrease of

neurotransmitters secreted by these nerve fibers. Therefore, expression of dopamine, noradrenaline and adrenaline was of interest.

3.2.4 Expression of noradrenergic neurotransmitters in peritoneal fluid is unmodified

Dopamine

Expression of dopamine in peritoneal fluid of women with endometriosis is not modified when compared to healthy women. (mean DOP ng/ml: 0.15±0.01 and 0.11±0.04 respectively. Mann-Whitney test. p = 0.55) (Fig. 12). Dopamine expression was independent of menstrual cycle phase or endometriosis stage (Tab. 1).

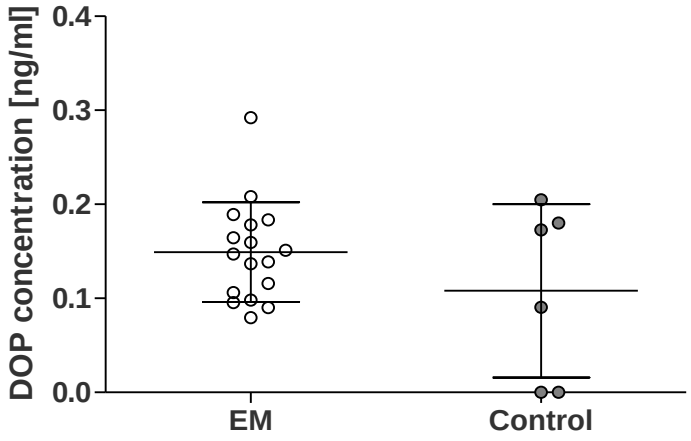


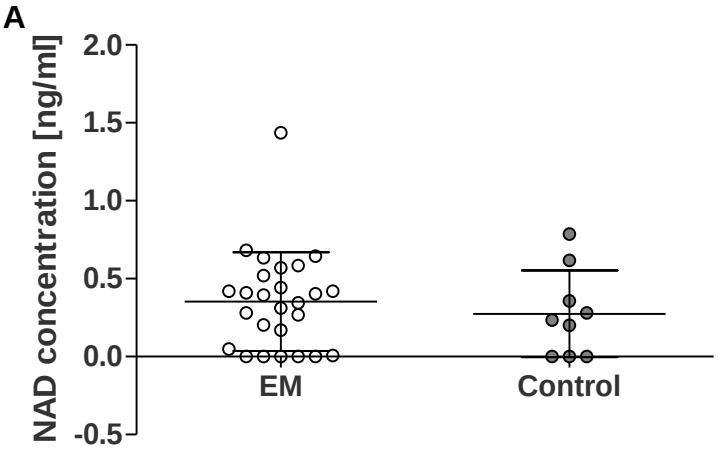
Figure 12: Dopamine concentration in peritoneal fluid of endometriosis patients (EM) and women without endometriosis (Control).

	EM stage	Dopamine [ng/mL]	cycle phase	Dopamine [ng/mL]
Peritoneal fluid	rASRM I	0.16 ± 0.01	proliferative	0.12 ± 0.03
	rASRM II	0.18 ± 0.06	secretory	0.17 ± 0.04
	rASRM III	0.14 ± 0.05	menses	0.18 ± ND
	rASRM IV	0.14 ± 0.02	OC	0.14 ± 0.02

Table 1: Mean concentration of dopamine in peritoneal fluid of women with endometriosis in different rASRM stages or menstrual cycle phase. (OC = intake of oral contraceptiva). No significant differences (Kruskal-Wallis test with post Dunn's multiple comparison test).

Noradrenaline

Noradrenaline (NAD) concentration is similar in peritoneal fluid of women with endometriosis or without endometriosis (mean NAD ng/ml: 0.35±0.06 and 0.28±0.09 respectively. Mann-Whitney test. p = 0.43) (Fig. 13 A). Comparison of NAD expression in different cycle phases or in women during intake of oral contraceptive (OC), did not result in any significant difference (mean NAD ng/ml, proliferative: 0.40±0.20, secretory: 0.21±0.10, menses: 0.17±0.07 and OC: 0.40±0.06 respectively. ND: no data. Kruskal-Wallis test p = 0.24). Nevertheless, NAD concentration seems to be slightly higher during intake of oral contraceptiva (Fig 13B). Similarly, when comparing concentration of NAD during different endometriosis stages, no significant difference could be determined (mean NAD ng/ml: rASRMI 0.37±0.06, rASRMII 0.32±0.11, rASRMIII 0.98±0.46, rASRMIV 0.25±0.09. Kruskal-Wallis test p = 0.29) (Fig. 13 C).



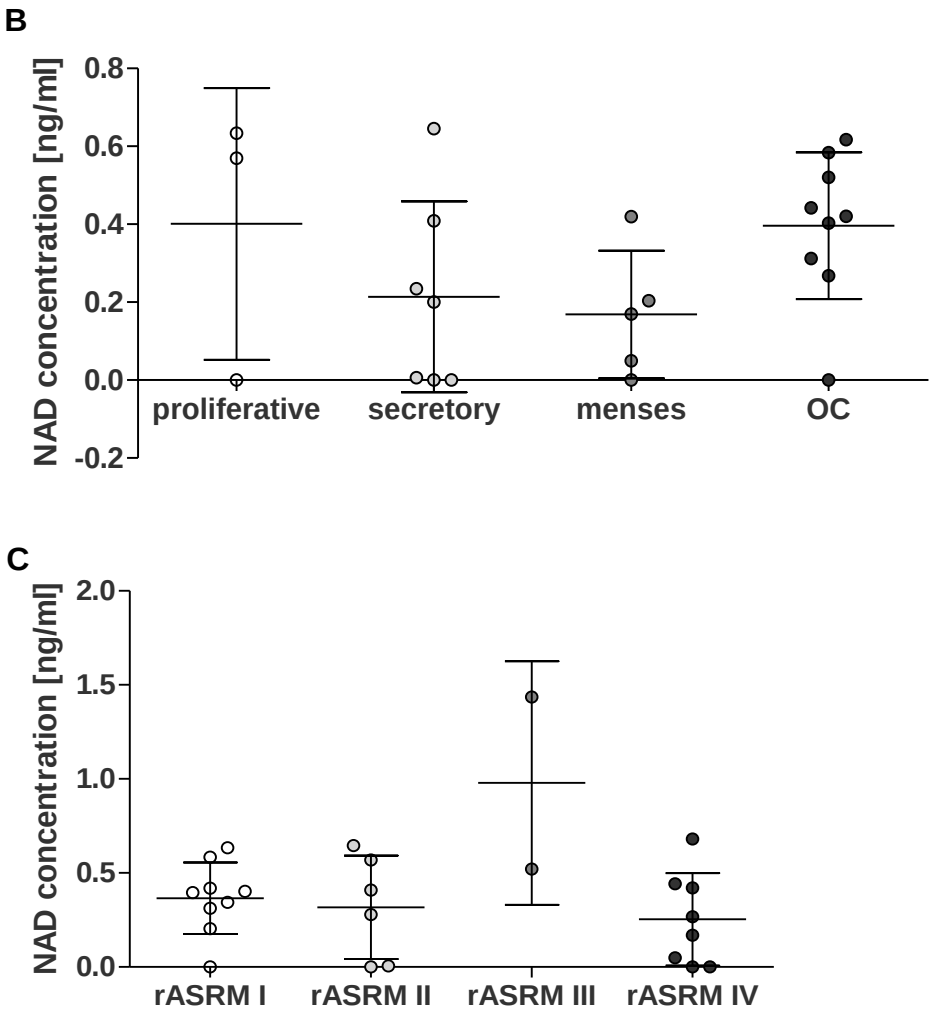


Figure 13: Mean concentration of noradrenaline in (A) peritoneal fluid of women with endometriosis (EM) and without endometriosis (Control); (B) in different menstrual cycle phases (OC = intake of oral contraceptiva) and (C) different rASRM stages.

Adrenaline

Adrenaline expression could not be identified in any sample independent of disease or healthy status.

3.2.5 Expression of cytokines in peritoneal fluid

Interleukin-4

The concentration of interleukin-4 (IL-4) in healthy or endometriosis patients remains unmodified (mean IL-4 pg/ml: 0.39±0.08 and 0.32±0.07 respectively. Mann-Whitney test. p = 0.75) (Fig. 14). Thereby, IL-4 concentration did not seem to be influenced by cycle phase of endometriosis stage (Tab. 2).

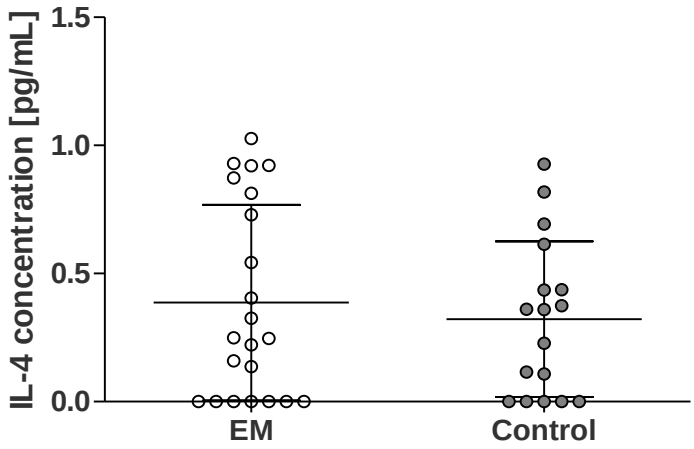


Figure 14: IL-4 concentration in peritoneal fluid of endometriosis patients (EM) and women without endometriosis (Control).

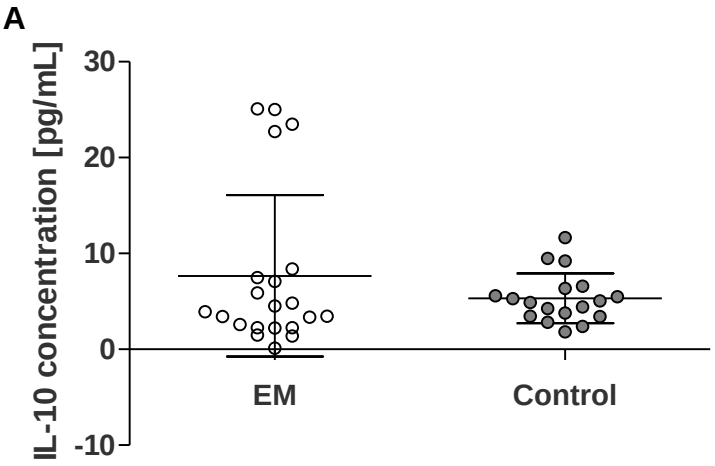
	EM stage	IL-4 [pg/mL]	cycle phase	IL-4 [pg/mL]
Peritoneal fluid	**rASRM I**	0.22 ± 0.13	**proliferative**	0.55 ± 0.15
	rASRM II	0.73 ± ND	**secretory**	0.25 ± 0.18
	rASRM III	0.35 ± 0.19	**menses**	ND
	rASRM IV	0.43 ± 0.20	**OC**	ND

Table 2: Mean concentration of IL-4 in peritoneal fluid of women with endometriosis in different rASRM stages or menstrual cycle phases (OC = intake of oral contraceptiva). No significant difference in IL-4 expression in rASRM stages (Kruskal-Wallis test with post Dunn's multiple comparison test). Proliferative and secretory cycle phase show no significant difference (Mann Whitney test p = 0.28).

Interleukin-10

The anti-inflammatory cytokine interleukin-10 (IL-10) is similarly expressed in peritoneal fluid of women with and without endometriosis (mean IL-10 pg/ml: 7.66±1.84 and 5.32±0.61 respectively. Mann-Whitney test. p = 0.56) (Fig. 10 A).

Expression of IL-10 is modulated during the cycle phase of healthy women, since it is responsible for wound healing during the cycle phase. This phenomenon could be evidence in our samples (mean IL-10 pg/ml: 7.48±1.31 and 4.20±0.24 in proliferative and secretory cycle phase respectively. Mann-Whitney test. p = 0.03). Interestingly, this modulation appears to be lost in women with endometriosis (mean IL-10 pg/ml: 6.29±2.77 and 8.22±3.82 in proliferative and secretory cycle phase respectively. Mann-Whitney test. p = 0.62) (Fig. 10 B-C). The endometriosis stage did not influence the occurrence of IL-10 (mean IL-10 pg/ml: rASRMI 4.16±1.85, rASRMII 4.13±0.69, rASRMIII 2.99±0.76, rASRMIV 13.15±4.64. Kruskal-Wallis with post Dunn's multiple comparison test p = 0.18).



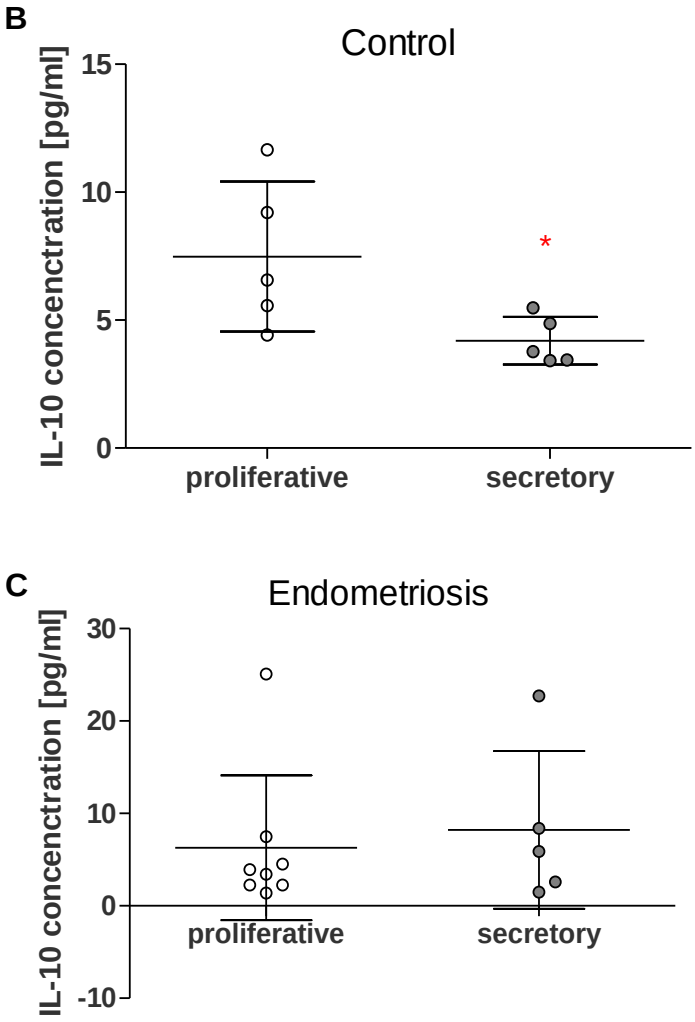


Figure 15: (A) IL-10 concentration in peritoneal fluid of women with (EM) and without endometriosis (Control). (B-C) Concentration of IL-10 in peritoneal fluid of endometriosis patients and control during the proliferative and secretory cycle phase respectively. *p<0.05

Interleukin-13

Expression of interleukin-13 (IL-13) could not be identified in any sample independent of disease or healthy status.

Interleukin-1β

Interleukin-1β (IL-1β) expression could not be identified in any sample independent of disease or healthy status.

TGF-β

TGF-β (transforming growth factor-β) expression could not be identified in any sample independent of disease or healthy status.

3.3 Serum analysis

3.3.1 Semas and Nrps expression in serum of endometriosis patients is unmodified

Concentration of Sema3C in serum of patients with endometriosis and women without endometriosis are similar (mean Sema3C ng/ml: 19.43±3.00 and 21.68±3.21 respectively. Mann-Whitney test. p = 0.5593). Furthermore, no differences in Sema3C concentration could be identified in dependence of cycle phase or endometriosis stage (Tab.3).

	EM stage	Sema3C [ng/mL]	cycle phase	Sema3C [ng/mL]
Serum	**rASRM I**	15.10 ± 4.90	**proliferative**	15.59 ± 5.93
	rASRM II	10.13 ± 2.12	**secretory**	29.47 ± 4.47
	rASRM III	22.05 ± 12.40	**menses**	ND
	rASRM IV	25.07 ± 5.55	**OC**	ND

Table 3: Mean Sema 3C concentration in serum of women with endometriosis in different rASRM stages or menstrual cycle phases (OC = intake of oral contraceptive, ND = no data). No significant difference in Sema 3C expression in rASRM stages (Kruskal-Wallis test with post Dunn's multiple comparison test p = 0.39). Menstrual cycle phase did not influence Sema 3C concentration in serum (Mann Whitney test. p = 0.20).

Sema3F expression in serum seems to be slightly lower in women with endometriosis than in women without endometriosis (mean Sema3F ng/ml: 983.3±72.64 and 1150±92.57 respectively. Mann-Whitney test. p = 0.08) (Fig. 16). When comparing the concentrations of Sema3F depending on cycle phase or endometriosis stage, no significant differences could be identified (Tab.4).

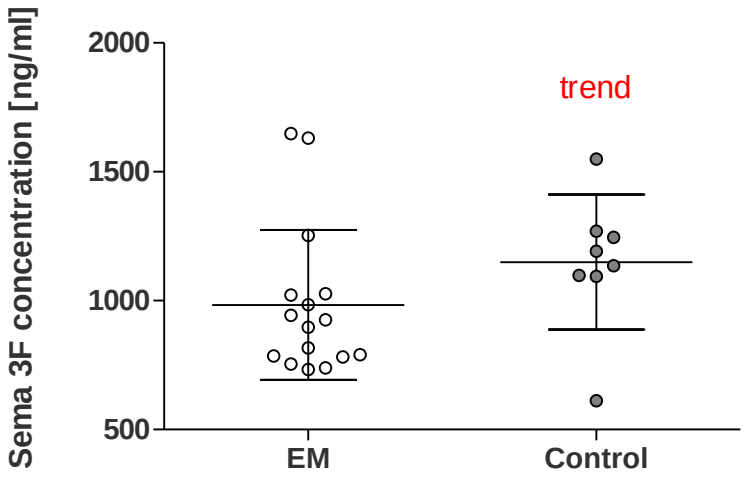


Figure 16: Sema 3F concentration in serum of endometriosis patients (EM) and women without endometriosis (Control).trend p<0.09

	EM stage	Sema3F [ng/mL]	cycle phase	Sema3F [ng/mL]
Serum	**rASRM I**	887.1 ± 36.11	**proliferative**	786.2 ± ND
	rASRM II	1648 ± ND	**secretory**	995.8 ± 136
	rASRM III	880.5 ± 140.9	**menses**	ND
	rASRM IV	1029 ± 121.2	**OC**	995.9 ± 211.8

Table 4: Mean Sema 3F concentration in serum of women with endometriosis in different rASRM stages or menstrual cycle phases (OC = intake of oral contraceptive, ND = no data). No significant difference in Sema 3F expression in rASRM stages (Kruskal-Wallis test with post Dunn's multiple comparison test p = 0.36). Menstrual cycle phase or OC did not influence Sema 3F concentration in serum Kruskal-Wallis test with post Dunn´s multiple comparison test p = 0.54).

Nrp1 expression is unmodified in serum of women with endometriosis (mean Nrp1 ng/ml: 19.43±3.00 and 21.68±3.21 endometriosis patients and women without endometriosis respectively. Mann-Whitney test. p = 0.71).

Similarly, expression of Nrp2 in serum remains unmodified when diseased with endometriosis (mean Nrp2 ng/ml: 21.07±3.61 and 27.91±7.54 endometriosis patients and women without endometriosis respectively. Mann-Whitney test. p = 0.41). Also during different menstrual cycle phases or with intake of oral contraceptive, Nrp2

expression remained stable. Endometriosis stage also did not influence Nrp2 expression in serum (Tab. 5).

	EM stage	Nrp2 [ng/mL]	cycle phase	Nrp2 [ng/mL]
Serum	rASRM I	24.67 ± 12.40	proliferative	10.20 ± 6.10
	rASRM II	16.48 ± 10.35	secretory	32.55 ± 14.69
	rASRM III	21.32 ± 11.75	menses	ND
	rASRM IV	18.24 ± 2.50	OC	18.86 ± 5.85

Table 5: Mean Nrp 2 concentration in serum of women with endometriosis in different rASRM stages or menstrual cycle phases (OC = intake of oral contraceptive, ND = no data). No significant difference in Nrp 2 expression in rASRM stages (Kruskal-Wallis test with post Dunn's multiple comparison test p = 0.99). Menstrual cycle phase did not influence Nrp 2 concentration in serum (Mann Whitney test. p = 0.33).

3.3.2 Neurotransmitters levels in serum during endometriosis is unaffected

Dopamine

Expression of dopamine in serum remains unmodified with endometriosis. No significant dopamine concentration could be identified in serum probes.

Noradrenaline

Noradrenaline (NAD) expression is similar in serum of women with and without endometriosis (mean NAD ng/ml: 0.78±0.10 and 0.55±0.12 respectively. Mann-Whitney test. p = 0.37).

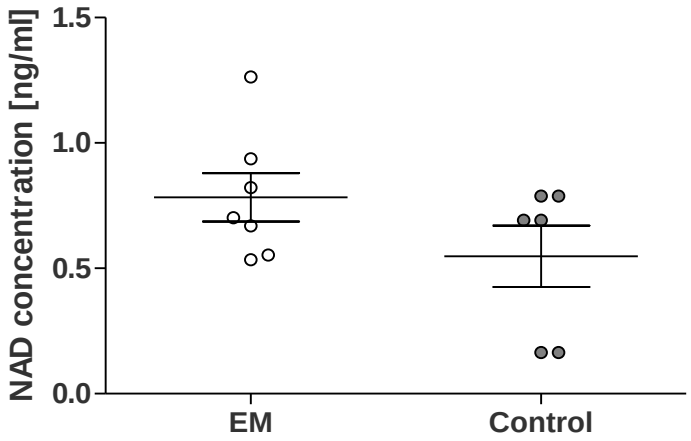


Figure 17: Noradrenaline concentration in serum of endometriosis patients (EM) and women without endometriosis (Control).

Adrenaline

Adrenaline (ADR) expression in serum of endometriosis patients and women without endometriosis was similar (mean ADR ng/ml: 0.07±0.02 and 0.07±0.03 respectively. Mann-Whitney test. p = 0.75).

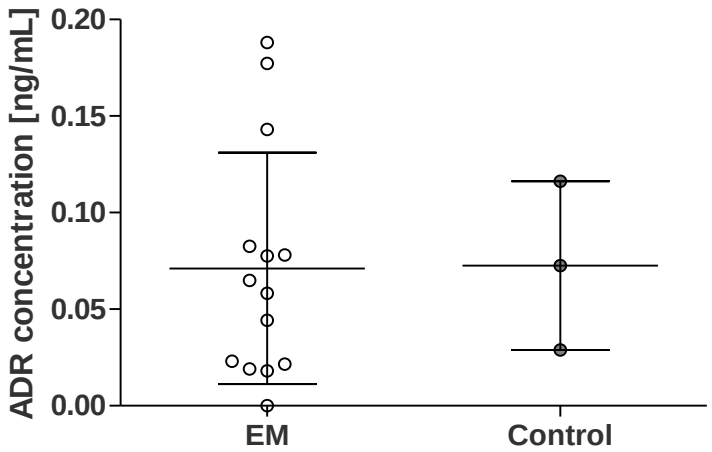


Figure 18: Adrenaline concentration in serum of endometriosis patients (EM) and women without endometriosis (Control).

3.3.3 Expression of cytokines in serum

Interleukin-4

IL-4 expression in serum of women with endometriosis is similar to the expression in serum of women without endometriosis (mean IL-4 ng/ml: 0.44±0.17 and 0.86±0.30 respectively. Mann-Whitney test. p = 0.34). Also when comparing different endometriosis stages no significant difference could be identified (mean IL-4 ng/ml: rASRMI 0.28±0.16, rASRMII 0±ND, rASRMIII 0.55±ND and rASRMIV 0.63±0.37.Kruskal-Wallis test. p = 0.57). Interestingly, concentration of IL-4 is slightly reduced in women with endometriosis, which experience pelvic pain in comparison to women without endometriosis, which do not suffer from pelvic pain (Fig. 19 A). Comparison of endometriosis patients with and without pelvic pain as well suggest reduced amounts of IL-4 in the patients who suffer from pelvic pain (Fig. 19 B).

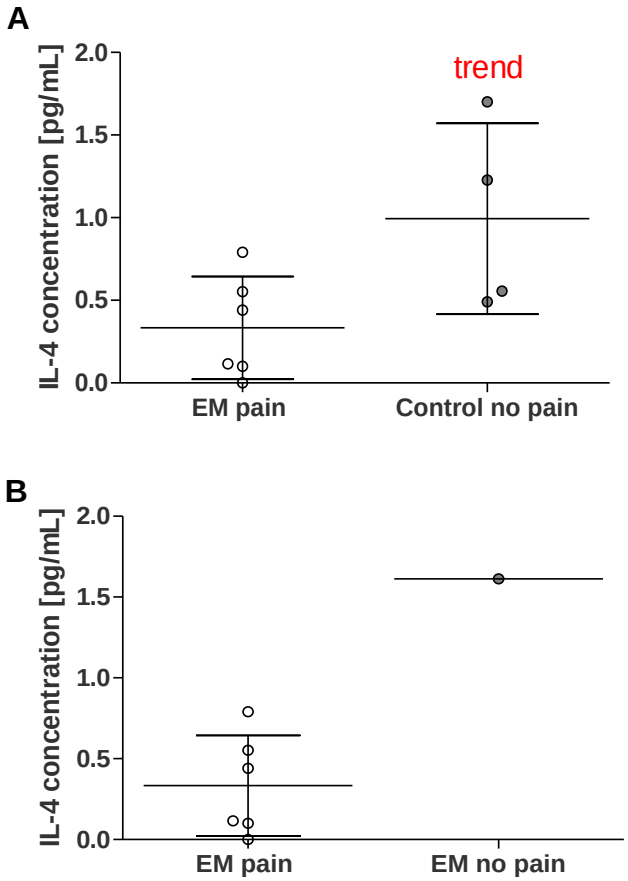


Figure 19: (A) IL-4 concentration in serum of women with endometriosis suffering from pelvic pain (EM pain), controls without pelvic pain (without endometriosis), and (B) patients with endometriosis without pelvic pain (EM no pain).

Interleukin-10

Expression of IL-10 in serum remains similar with or without endometriosis (mean IL-10 ng/ml: 6.70±1.17 and 7.20±1.19 respectively. Mann-Whitney test. p = 0.54). Comparison of concentrations in endometriosis patients suffering from pelvic pain with non-endometriotic women without pelvic pain, revealed no significant difference (mean IL-10 ng/ml: 6.49±1.30 and 6.49±0.97 respectively. Mann-Whitney test. p = 0.52). Endometriosis stage neither played a role in the concentration of IL-10 (mean IL-10 ng/ml: rASRMI 5.74±1.54, rASRMII 2.99±ND, rASRMIII 13.18±ND and rASRMIV 7.16±1.52.Kruskal-Wallis test. p = 0.21).

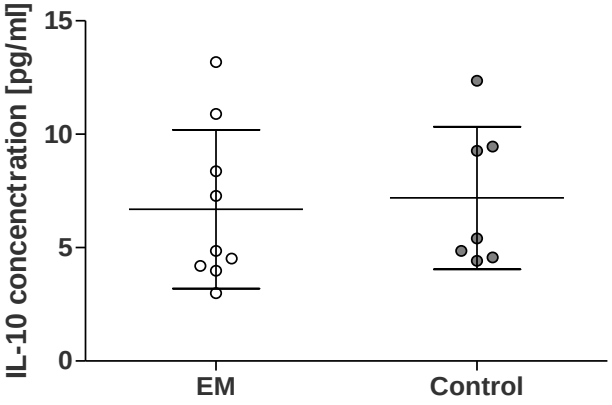


Figure 20: IL-10 concentration in serum of women with endometriosis (EM) and without endometriosis (Control).

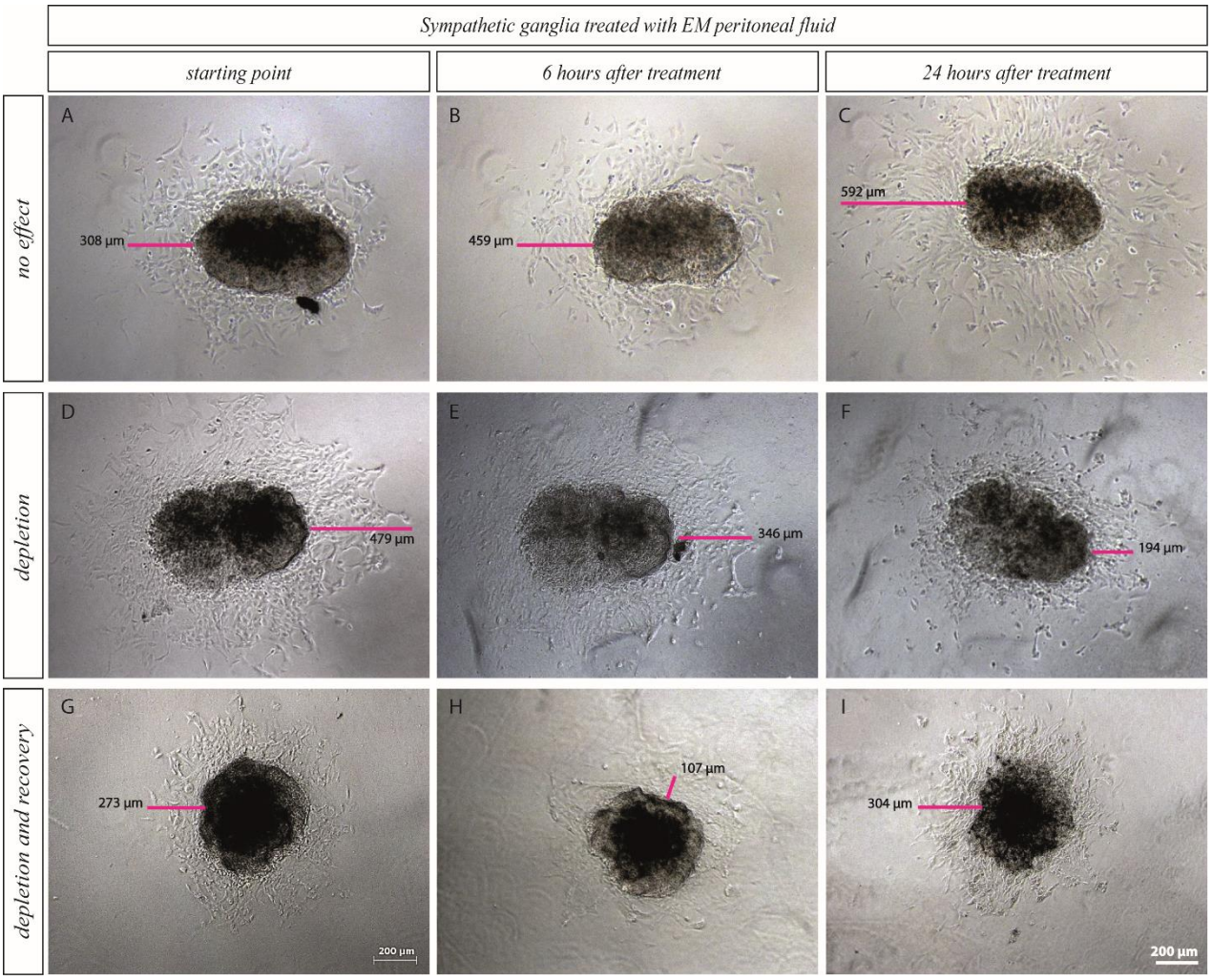
Interleukin-1β

Expression of IL-1β could not be identified in any sample independent of disease or healthy status.

3.4 Analysis of in vitro effects of Semas and peritoneal fluid on ganglia

3.4.1 Incubation of sympathetic chain ganglia with peritoneal fluid of endometriosis patients can lead to axon growth collapse

Sympathetic ganglia are affected when incubated with peritoneal fluid of women with endometriosis. Thereby, three different reactions could be observed. In three out of twelve cases, the incubation of the ganglia with peritoneal fluid of women with endometriosis led to inhibition of further axonal outgrowth after 6 hours and consequently to depletion of axons after 24 hours (Fig. 21 D-F). In eight of twelve cases, inhibition of further axonal outgrowth could be identified; nevertheless, after 24 hours the ganglia seem to recover and axons continued growing (Fig. G-I). Further, in one case, no effect could be seen (Fig. 21 A-C). Incubation of sympathetic ganglia with peritoneal fluid of women without endometriosis, led in two out of three cases to no effect and in one case to an inhibition of further outgrowth after 6 hours and a recovery of the outgrowth after 24 hours (Fig. 21 J-L).



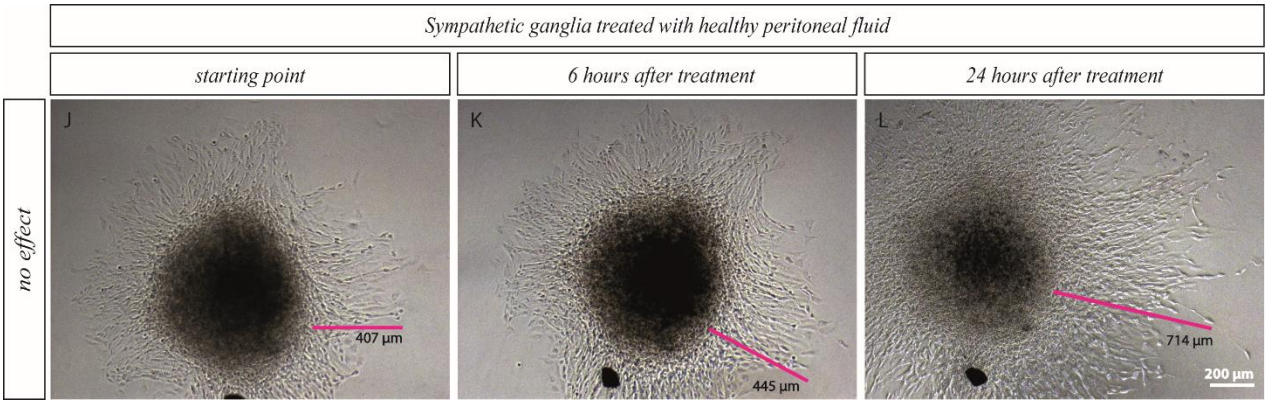


Figure 21: Sympathetic ganglia form chicken embryo. Incubated for 24 hours in cell culture medium (A, D, G, J), and treated with peritoneal fluid of women with endometriosis for 6 hours (B, E, H) and 24 hours (C, F, I); or without endometriosis (healthy peritoneal fluid) for 6 hours (K) and 24 hours (L). Pink line shows the length of the axonal outgrowth.

3.4.2 Incubation of sympathetic chain ganglia with Sema3C and Sema3F induces axon growth collapse

Sympathetic ganglia incubated for 24 hours show sympathetic outgrowth. Consequently, ganglia were treated with Sema3C and 3F.

Sema3C and Sema3F elicited nerve fiber repulsion of sympathetic ganglia outgrowth after 6 hours. Nevertheless, the repulsion did not lead to a complete depletion of axonal outgrowth. After 24 hours of incubation with semas, axons seem to recover and started growing again (Fig. 22).

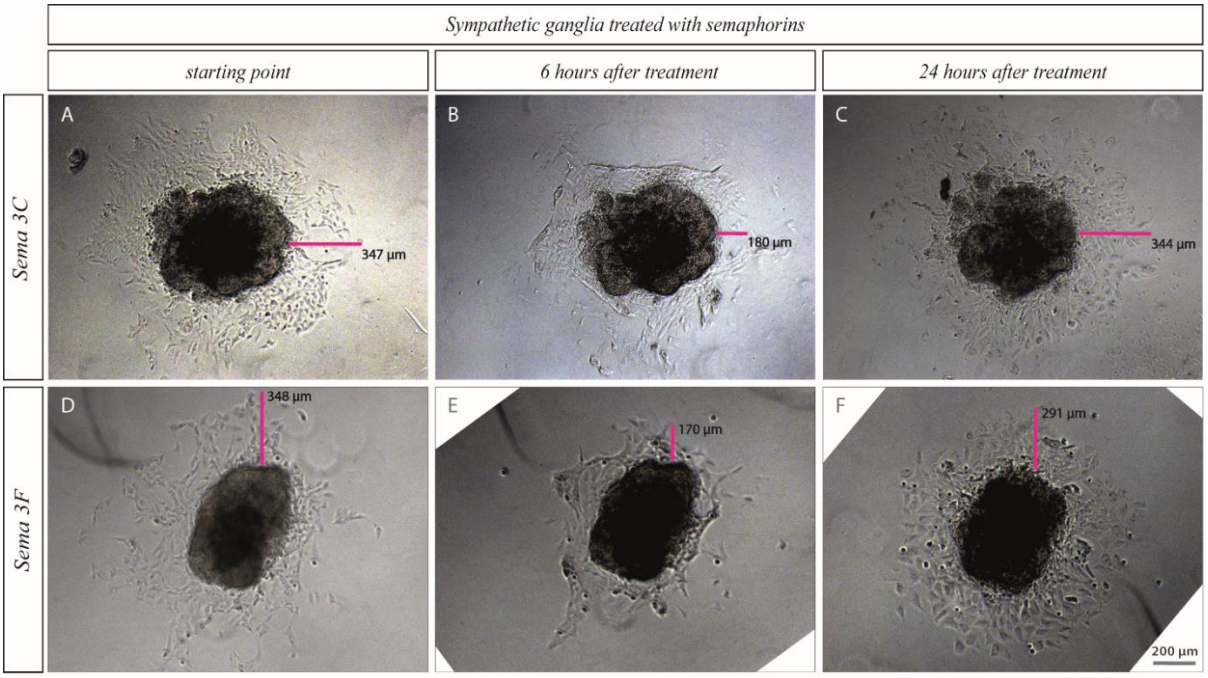


Figure 22: Sympathetic ganglia form chicken embryo. Incubated for 24 hours in cell culture medium (A, D), and treated with Sema 3C (B-C); or Sema 3F (E-F) for for 6 hours and 24 hours respectively.

3.4.3 Semas antibody can partially inhibit peritoneal fluid effects on sympathetic ganglia

Sympathetic ganglia outgrowth are repelled by peritoneal fluid, addition of Sema3C or Sema3F antibody was able to prevent axonal growth collapse. Nevertheless, the axons would not continue growing after treatment with peritoneal fluid and Sema antibody. However, these treatments stressed the ganglia severely, so that after approximately 24 hours they would start becoming apoptotic (Fig. 23).

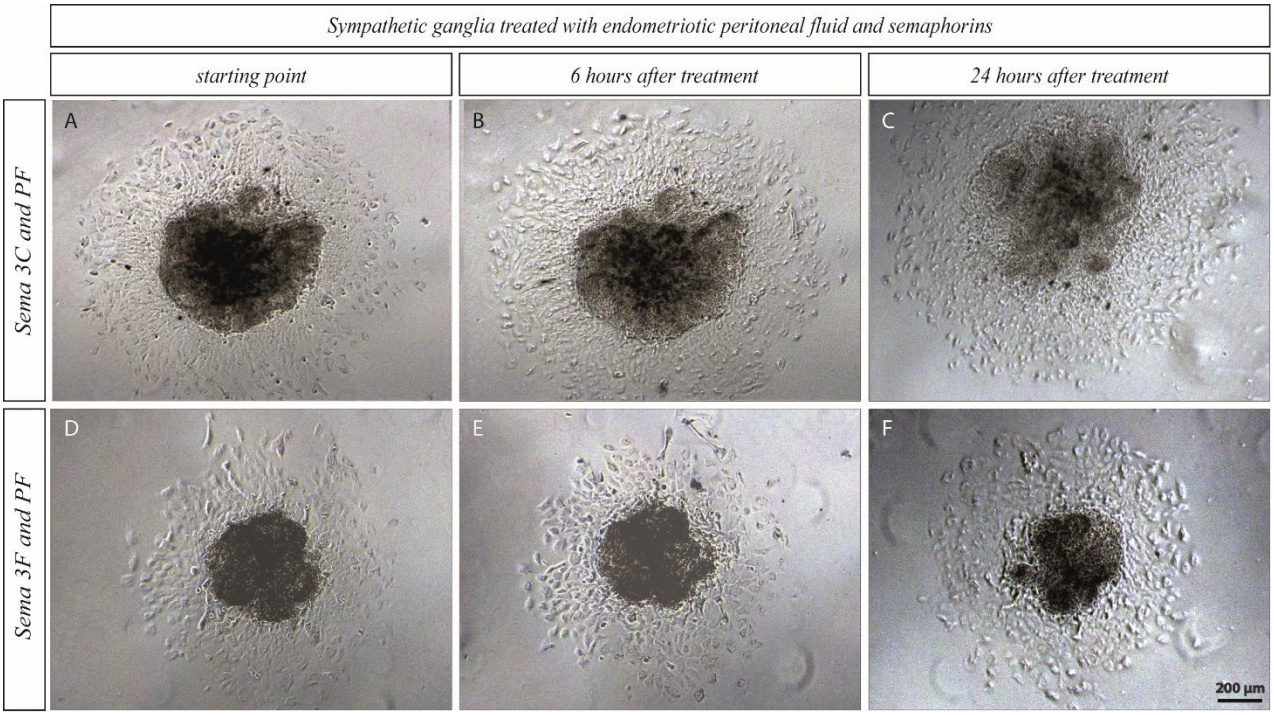
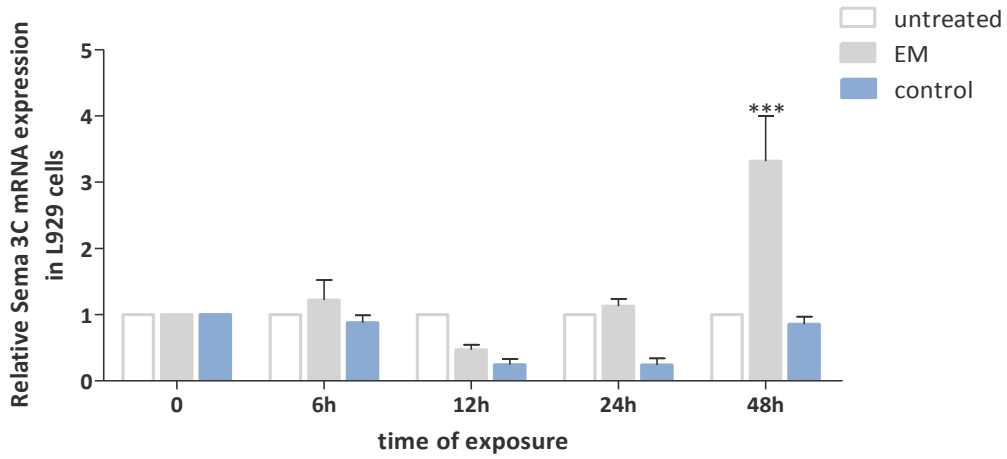


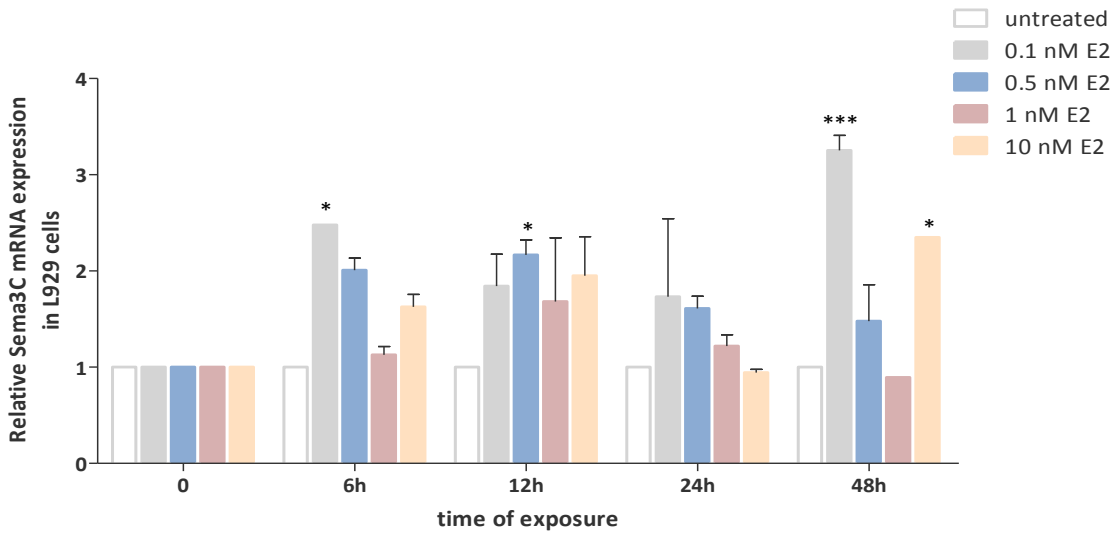
Figure 23: Sympathetic ganglia from chicken embryo. Treated for 6 hours and 24 hours with peritoneal fluid of women with endometriosis and Sema 3C and Sema 3F (D-F).

3.5 Analysis of in vitro effects of peritoneal fluid

3.5.1 Sema and Nrp expression can be modulated by estrogen, TGFβ and peritoneal fluid of women with endometriosis

Treatment of fibroblasts with estrogen (E2) resulted in a significant increase of Semas and Nrp2 expression after six hours. Similarly, treatment with TGFβ, lead to an increase of Semas and Nrp2 after six hours. When treating the cells with peritoneal fluid of women with endometriosis, Sema3C expression was increased after 48 hours; Nrp2 expression already after 24 hours. Interestingly, expression of Sema3F is not only modulated by peritoneal fluid of women with endometriosis, but also by peritoneal fluid of women without endometriosis (Fig. 24-26).





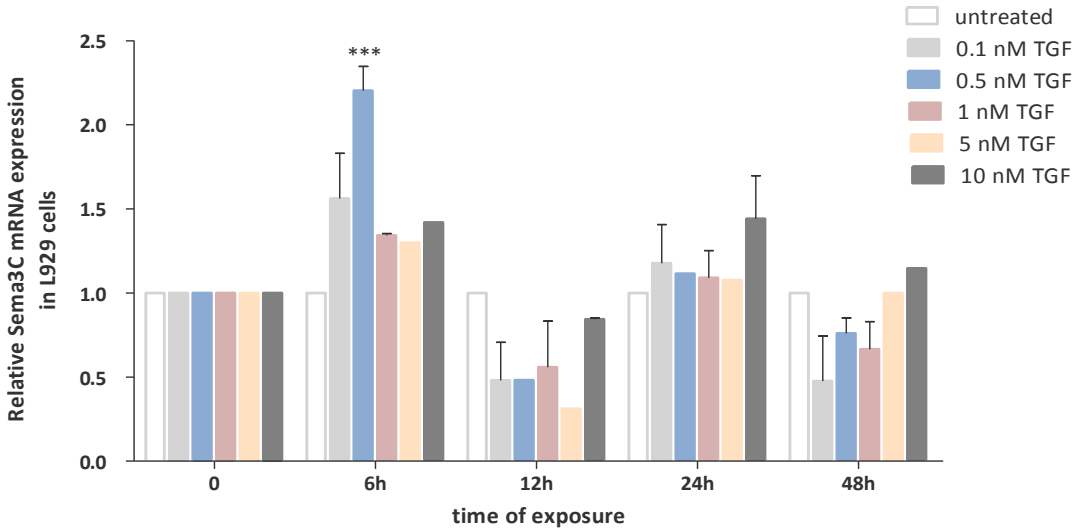


Figure 24: Relative Sema 3C expression in L929 cells, after treatment with peritoneal fluid (A), estrogen (B) or TGF (C).*p<0.05; ***p<0.001

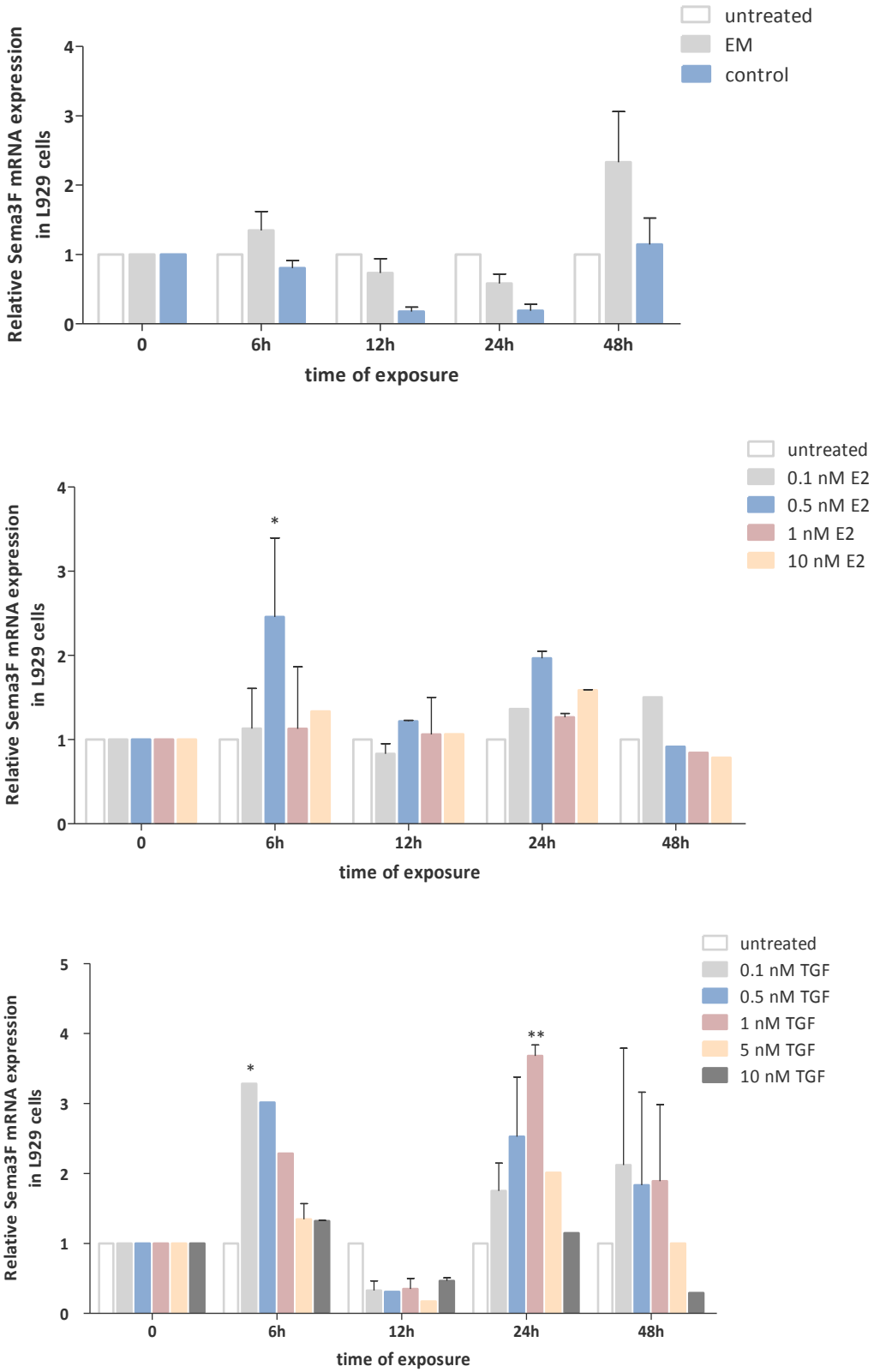


Figure 25: Relative Sema 3F expression in L929 cells, after treatment with peritoneal fluid (A), estrogen (B) or TGF (C).*p<0.05;**p<0.01

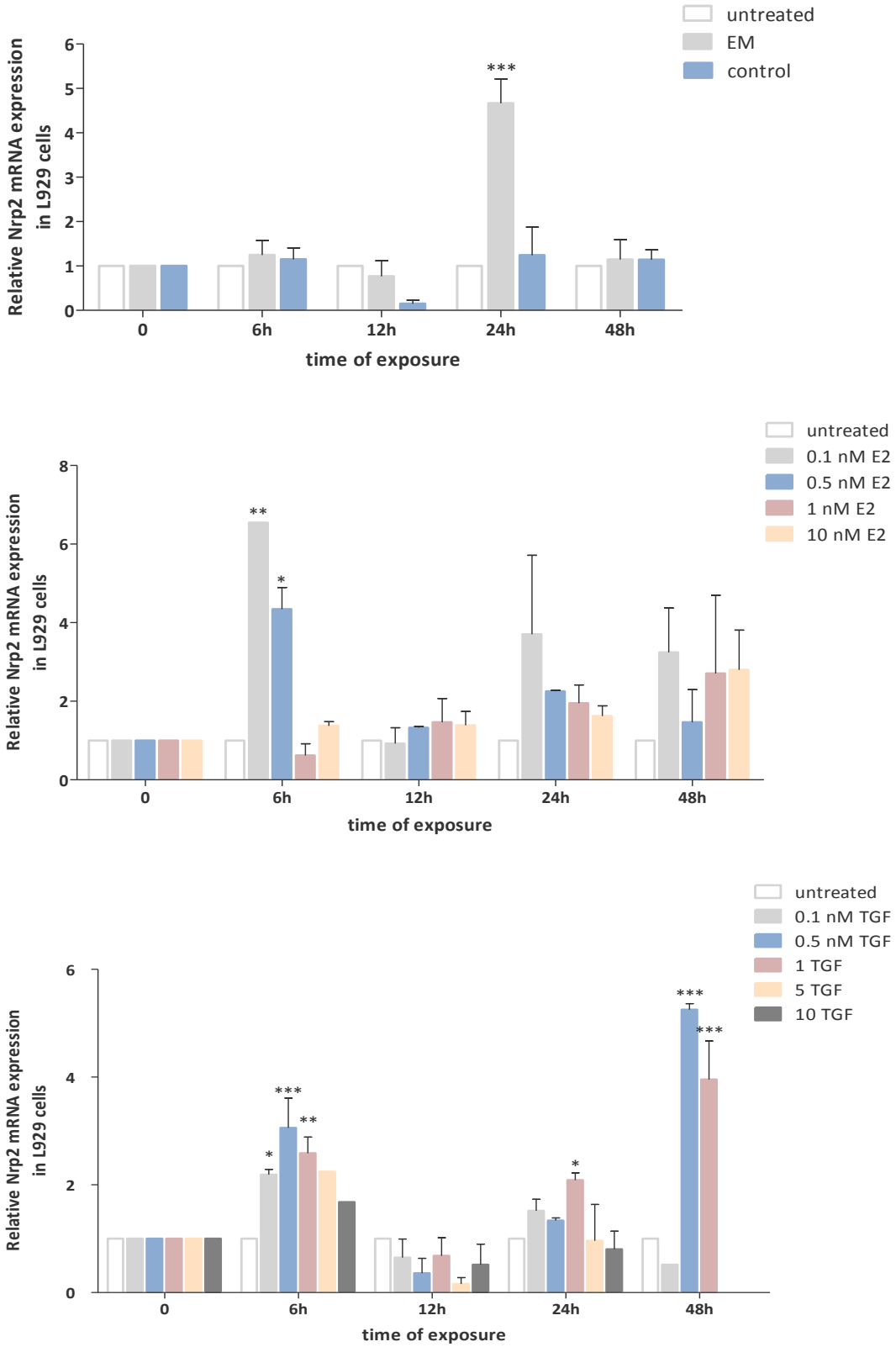


Figure 26: Relative Sema 3C expression in L929 cells, after treatment with peritoneal fluid (A), estrogen (B) or TGF (C).*p<0.05; **p<0.01; ***p<0.001

4 Discussion

Neurotrophic actions of endometriosis lesions lead to modification in the innervation, potentially contributing to pain emergence during endometriosis (Anaf, et al., 2002, Mechsner, et al., 2007). Similarly, the reduced amounts of sympathetic nerve fibers in peritoneal endometriosis, which could be verified in this study.

Specific semaphorins, which have already been suggested as potential factors leading to a decrease of sympathetic nerve fibers in other chronic inflammatory diseases such as rheumatoid arthritis (Miller, Weidler, Falk, Angele, Schaumburger, Scholmerich and Straub, 2004), might also be involved in sympathetic nerve fiber depletion during endometriosis. In this study an associative negative correlation between Semaphorin expression and sympathetic nerve fiber occurrence in peritoneal tissue of women with endometriosis could be demonstrated. Furthermore, these sympathetic nerve fibers express the main Sema receptors. Interestingly, even though sympathetic nerve fibers are reduced in affected as well as unaffected peritoneum of women with endometriosis, Sema expression was only elevated in affected peritoneum. Nevertheless, these nerve fibers were in both cases positive for Sema receptors. The increase of Sema3C and soluble Nrp2 in the peritoneal fluid, which could be demonstrated, might influence the overall occurrence of sympathetic nerve fibers in the peritoneal cavity of endometriosis patients. Furthermore, it could be demonstrated that the peritoneal fluid of endometriosis patients is able to induce Sema and Nrp2 expression in vitro. Besides, the repellent effects of peritoneal fluid on sympathetic ganglia outgrowth was demonstrated, and partly attributed to Sema3C and 3F expression.

The reduced occurrence of sympathetic nerve fibers in the peritoneal cavity, might lead to some discrepancies in the balance of neurotransmitters which act anti-inflammatory. Therefore, the expression of these was analyzed in peritoneal fluid, revealing no changes in the expression of neurotransmitters secreted by sympathetic nerve fibers. In the body there are many important sources for these neurotransmitters, (e.g. the adrenal gland) and these seem to compensate the expression of neurotransmitters in the peritoneal fluid. Nevertheless, the actions of these specific neurotransmitters occurs locally, therefore the reduction of sympathetic nerves in specific regions probably still impairs the affected regions. The expression of further pro- and anti-

inflammatory acting cytokines remains unmodified in peritoneal fluid. During endometriosis chronic inflammatory processes dominate.

The fact that anti-inflammatory acting factors are not elevated, suggests dysfunctional immune response mechanisms during endometriosis, probably contributing to the chronification of the disease.

The aim of this work was to identify factors, which potentially lead to the decrease of sympathetic nerve fibers in peritoneal endometriosis. Further, the effects of the decrease on the overall immune response and neuroimmune mechanisms during endometriosis were studied.

4.1 Endometriosis reduced sympathetic innervation – potential causes and effects

A known phenomenon in chronic inflammatory diseases, as well as in endometriosis, is the poor sympathetic innervation (Ferrero, et al., 2010) (Arnold, Barcena de Arellano, Ruster, Vercellino, Chiantera, Schneider and Mechsner, 2012, Miller, Justen, Scholmerich and Straub, 2000, Weidler, Holzer, Harbuz, Hofbauer, Angele, Scholmerich and Straub, 2005). In this study, a significant reduction of sympathetic nerve fibers could be demonstrated in peritoneal endometriosis. However, the cause for this reduction remains unclear. Semaphorins class 3, represent a potential factor modulating the innervation during inflammatory diseases or even just disease (Binch, et al., 2015, Fassold, Falk, Anders, Hirsch, Mirsky and Straub, 2009). Therefore, in this study we demonstrated an associative negative correlation between Sema3C and 3F expression and occurrence of sympathetic nerve fibers in affected endometriotic tissue. Here, the identified nerve fibers were positive for the main Sema receptors, responsible for axon repulsion actions, such as Nrp1, Nrp2 and Plexin A3 and A4 (Suto, et al., 2005, Waimey, Huang, Chen and Cheng, 2008, Yaron, Huang, Cheng and Tessier-Lavigne, 2005). Therefore, the repellence of these nerve fibers would be possible through effects of the identified expression of Sema 3C and 3F in this tissue. Importantly, the Sema receptor expression is normally not expected to be found on adult nerve fibers, but rather during pathological or developmental events. Therefore, the expression of these receptors on TH-positive endometriosis associated nerve fibers, points at severe changes in the modulation of these nerves and potentially

severe nerve damage. Unexpectedly, even if sympathetic nerve fibers occurrence was reduced in the affected and unaffected peritoneum of women with endometriosis, Sema expression was not elevated in the unaffected peritoneum. The occurrence of macrophages and activated fibroblasts was not elevated in the unaffected peritoneum and since Sema3C and 3F was demonstrated to be mostly expressed by these two types of cells, the low expression of Semas in unaffected peritoneum in women with endometriosis, was attributed to the lack of macrophages and activated fibroblasts in that tissue. Macrophages and activated fibroblasts are not solely responsible for Sema expression. Endometrial stromal and endothelial cells also showed Sema expression. By RNA in situ hybridization, Sema expression at RNA level could be identified near endometriosis lesions, suggesting that Sema is synthesized by cells surrounding endometriosis lesions. However, RNA expression of Semas could not be identified in unaffected peritoneum of women with endometriosis, indicating that only endometriosis lesion associated cells, macrophages and activated fibroblasts are able to express Sema3C and 3F.

By these results, the reduction of sympathetic nerve fibers in the unaffected peritoneum of women with endometriosis, could not be associated with Sema3C or 3F expression. Yet, the sympathetic nerve fibers identified in the unaffected and affected peritoneum were similar; i.e. they both expressed the Sema receptors at their surface, a property of these nerve fibers which could not be seen in the nerve fibers identified in peritoneal tissue of women without endometriosis. The expression of the Sema receptors, provides the possibility of repellence by Semas. Analysis of peritoneal fluid revealed an increased concentration of Sema3C only in women with endometriosis. Since the peritoneal fluid is in constant contact with the whole peritoneum, it would suggest that the Sema3C present in the peritoneal fluid could repel Nrp/Plexin-positive nerve fibers in the unaffected peritoneum of women with endometriosis. Furthermore, soluble Nrp2 was demonstrated to be present in significantly higher concentrations in peritoneal fluid of women with endometriosis. Studies in rheumatoid arthritis have shown that soluble Nrp2 contributes and even aggravates sympathetic nerve fiber repulsion and arthritis score in a mouse model, suggesting as well an aggravation of the inflammation (Fassold, Falk, Anders, Hirsch, Mirsky and Straub, 2009). Therefore, the high levels of Nrp2 in peritoneal fluid of women with endometriosis, might also contribute to the repellence of sympathetic nerve fibers in the whole peritoneum and possibly even affect the inflammatory process. Interestingly, Nrp2 expression in peritoneal fluid

seemed to be slightly correlated with endometriosis stage and occurrence of pelvic pain. Women with higher endometriosis stage and suffering from pelvic pain, tended to have higher concentrations of Nrp2. These results, might corroborate that Nrp2 affects the inflammatory mechanisms and thereby probably also the pain emergence.

Importantly, the sympathetic nervous system is known to have multifactorial functions during inflammation. Depending on the neurotransmitters it releases, the concentration of the neurotransmitters and timing of activity in relation to the inflammatory course, it might act pro- or anti-inflammatory. Particularly during the early inflammatory response, the sympathetic nervous system is crucial and acts pro-inflammatory (Levine, et al., 1988, Levine, et al., 1987). It is known that low levels of noradrenaline augment inflammatory processes and high levels act anti-inflammatory (Flierl, et al., 2007, Riepl, et al., 2010). Therefore, the reduced amount of sympathetic nerve fibers in peritoneal tissue of women with endometriosis, might indicate low release of noradrenergic neurotransmitters and thereby induce pro-inflammatory effects. Interestingly, even if sympathetic nerve fibers are reduced in the peritoneum of women with endometriosis, the dopamine, noradrenaline and adrenaline levels seem to be similar in women with or without endometriosis. Probably release of the noradrenergic neurotransmitters is compensated by the adrenal gland. Indeed, the reduction of the neurotransmitters might acutally be down regulated, though locally, i.e. in the regions where the TH-positive nerve fibers are missing. Furthermore, low levels of noradrenaline act through α-adrenergic receptors, stimulating pro-inflammatory factors in neutrophils and macrophages (Flierl, Rittirsch, Nadeau, Chen, Sarma, Zetoune, McGuire, List, Day, Hoesel, Gao, Van Rooijen, Huber-Lang, Neubig and Ward, 2007, Spengler, et al., 1990). Since in this study we demonstrated significantly higher occurrence of macrophages in endometriotic tissue, the low levels of noradrenaline might induce an even more critical pro-inflammatory response.

During a typical inflammatory process, noradrenaline production becomes active, due to its immunosuppressive actions. This mechanism seems to be impaired in endometriosis, where no elevated levels of noradrenaline in peritoneal fluid could be found. Since noradrenaline act locally, a reduction of noradrenaline secretion near EM lesions might probably lead to low concentrations of noradrenaline in the affected tissue and thereby promotion of the pro-inflammatory actions of noradrenaline (Elenkov, Wilder, Chrousos and Vizi, 2000). In this study, slightly higher amounts of

noradrenaline could be identified during higher endometriosis stages, where more tissue and organs are affected. Possibly, organs such as the adrenal gland could then start responding to the inflammation, because of the extensive propagation of the endometriosis.

These findings suggest that the countering of the chronic inflammation in endometriosis might be impaired.

Serum analysis revealed no significant differences in Sema or Sema receptors expression. Sema3C and 3F are secreted proteins; therefore, they might be secreted from the peritoneal tissue cells into the peritoneal fluid. However, Semas are not able to reach the blood system. These findings elucidate that immune cells, which are transported through the vessels, first start expressing Semas when they are recruited to the site of inflammation or more specifically the endometriosis affected tissue. Also suggesting that factors capable of inducing Sema expression have to be present in the peritoneum and or peritoneal cavity of endometriosis patients.

In the case of the neurotransmitters no significant differences could be identified, as expected after the analysis of their expression in the peritoneal fluid.

The results of the serum analysis, lead to the assumption that endometriosis really is a disease affecting mostly the peritoneal cavity and not the entire system.

4.2 Dysfunctional pro- / anti-inflammatory events in endometriosis

Since a reduction of sympathetic innervation in peritoneal tissue of endometriosis affected women is known and could be verified in this study, the following step was to investigate if neurotransmitters, normally secreted by sympathetic nerve fibers, are reduced during endometriosis. However, the analysis of the expression levels of dopamine, noradrenaline and adrenaline in peritoneal fluid and serum led to no significant differences in expression levels when comparing samples from women with or without endometriosis. Other organs, such as the adrenal gland also produce noradrenergic neurotransmitters and probably even if the sympathetic nerve fibers are reduced in the peritoneum, the secretion can be compensated by organs such as the adrenal gland. Nevertheless, the expression of noradrenaline in peritoneal fluid was somewhat conspicuous, since slight differences in expression levels could be identified

when comparing women with or without hormonal treatment. In accordance to this result, women who are under hormonal treatment would get recovered from the sympathetic nerve fiber loss. This issue has to be further investigated with a higher amount of samples.

Tissues, which are not directly innervated by sympathetic nerve fibers, can be stimulated by catecolamines. Catecholamines are transported in the blood and reach organs and tissues throughout the body. Therefore, they have a much wider breadth of activity when compared to noradrenergic neurotransmitters (McCorry, 2007).

During endometriosis, the peritoneal cavity milieu is chronic inflamed; therefore, we expected to find reduced amounts of anti-inflammatory factors and increased amounts of pro-inflammatory factors in women with endometriosis. MMPs are known to lead to a degradation of the extra cellular matrix and promote invasion during endometriosis. IL-1 is an important pro-inflammatory factors, which induces MMPs expression (Starzinski-Powitz, Handrow-Metzmacher and Kotzian, 1999). Nevertheless, in our study, no significant difference in IL-1 expression could be found. Since MMPs regulation occurs cycle dependent, this might be a reason why we were not able to identify IL-1 expression in peritoneal fluid. Furthermore, MMP expression can be regulated by further inflammatory cytokines, such as IL-6 and TNF-α (Nap, 2012).

Another aspect of endometriosis, is its properties of autoimmune disease (Lebovic, Mueller and Taylor, 2001, Nothnick, 2001). IL-4 is an important regulator during autoimmunity and is therefore an important target during endometriosis. IL-4 is responsible for M2 macrophage activation during inflammation and wound repair. After M2 macrophage activation, IL-10 and TGF-β are secreted, resulting in decreased inflammation (Kiguchi, Kobayashi, Saika, Sakaguchi, Maeda and Kishioka, 2015, Lech and Anders, 2013). Studies in 2012 described an increase of IL-4 expression in serum and peritoneal fluid of adolescent girls suffering from pelvic pain than in controls without endometriosis (Drosdzol-Cop, Skrzypulec-Plinta and Stojko, 2012). In our study, we were not able to demonstrate any significance in IL-4 expression when comparing an endometriosis group with a non endometriosis group. Even more, we could demonstrate a trend of lower IL-4 expression in serum of women with endometriosis suffering form pelvic pain. In the study from Drosdzol-Cop the occurrence of endometriosis was not verified in the samples of the adolescent girls with pelvic pain, this might explain the difference of our results. Interestingly, IL-10 expression seems

to be regulated during the cycle phase. This could be shown in the controls without endometriosis, where IL-10 is higher expressed during the proliferative phase, probably contributing to wound healing, and lower during the secretory cycle phase. Importantly, this process is disturbed in women with endometriosis, there we could not identify any significant differences in the expression levels of IL-10 during different cycle phases, which suggests an impairment of the wound healing and repair system in women with endometriosis.

A further important cytokine during wound healing, tissue repair and fibrosis is TGF-β (Branton and Kopp, 1999). TGF-β is suggested to promote the ability of fibroblasts to contract collagen and thereby secure a provisional wound matrix. Additionally TGF-β is involved in fibroblast recruitment and activation (Montesano and Orci, 1988) (Kalluri and Zeisberg, 2006, Ronnov-Jessen and Petersen, 1993). Since in our study we were able to demonstrate acute activation of fibroblasts, we hypothesized an induction by TGF-β, nevertheless, no significant expression of TGF-β could be identified in peritoneal fluid. These results, suggest either an activation and recruitment of peritoneal fibroblasts by other mechanisms, or TGF-β is only expressed in tissue and not released into the peritoneal fluid.

Ultimately, no prominent significant in the investigated pro- and anti-inflammatory factors could be identified when comparing women with and without endometriosis. Nevertheless, the chronic inflammatory milieu is probably promoted by the lack of increase of important anti-inflammatory factos such as IL-4, IL-10 or TGF-β, which play a key role during wound healing and repair. Furtheremore, during our sample collection peritoneal fluid is centrifuged. Thereby many important immune cells, which might be positive for several pro and anti-inflammatory factors, are lost. Therefore, the results of expression of different cytokines is probably different when these cells are kept in the fluid.

4.3 Neurotrophic effects of the peritoneal fluid and the role of the semaphorins

Several studies have demonstrated expression of important factors, which regulate neurite outgrowth, in peritoneal, ovarian and rectovaginal endometriosis (Anaf, Simon, El Nakadi, Fayt, Simonart, Buxant and Noel, 2002, Borghese, et al., 2010, Mechsner, Schwarz, Thode, Loddenkemper, Salomon and Ebert, 2007). So, neurotrophic

properties have been attributed to endometriosis lesions. Importantly, endometriosis lesions affect the milieu in the peritoneal cavity in a way that has been hypothesized to affect the establishment, maintenance and symptoms of endometriosis. The factors secreted by endometriotic lesions include prostaglandins (Chishima, et al., 2007, Drake, et al., 1981), haptoglobin (Sharpe-Timms, 2005), cytokines e.g. IL-1, IL-6, IL-8 and IL-10; growth factors e.g. VEGF, NGF, TGF and IGF (Anaf, Simon, El Nakadi, Fayt, Simonart, Buxant and Noel, 2002, Gazvani and Templeton, 2002, Sharpe-Timms, 2001, Taylor, 2002). In this study, the expression of Semas, which are important neuromodulators, could be identified in higher levels the peritoneal fluid of women with endometriosis when compared to the control without endometriosis. Even if the effects of these factors and others, which are secreted by the lesions are not fully understood, the altered milieu in the peritoneal fluid is certainly crucial in the pathogenesis and pain genesis of endometriosis.

The presence of neurotrophic factors in the peritoneal fluid of women with endometriosis, suggest neurotrophic properties of the peritoneal fluid itself. In this study, we analyzed the effects of peritoneal fluid on sympathetic chain ganglia. The analysis revealed that the peritoneal fluid indeed is able to regulate sympathetic nerve fiber outgrowth. Interestingly, depending on the pelvic pain status of the endometriosis patients, the effects on the outgrowth were different. Peritoneal fluid from patients suffering from severe pelvic pain induced outgrowth inhibition and axonal collapse; peritoneal fluid from patients suffering from moderate pelvic pain or no pelvic pain induced solely outgrowth inhibition and no axonal cone collapse could be identified. These results suggest that the peritoneal fluid of endometriosis patients suffering from severe pelvic pain has higher neurotrophic properties than the peritoneal fluid of endometriosis patients with moderate or no pelvic pain at all.

Specific semaphorins are largely known for their neuromodulatory effects. Several studies have demonstrated sympathetic axon repulsion by semaphorins (Adams, et al., 1997, Kolodkin, 1996), which could be evidenced in this study. Recent studies have even associated the Sema receptor Nrp2 as factor aggravating nerve fiber repulsion (Fassold, Falk, Anders, Hirsch, Mirsky and Straub, 2009). In this study, significantly higher concentrations of Sema3C and Nrp2 could be identified in the peritoneal fluid of endometriosis patients. The expression of Semas and Nrps in tissue and fluid of endometriosis patients was negatively correlated with the sympathetic nerve fiber

occurrence in this study, suggesting a possible modulation of the sympathetic innervation by Semas. Therefore, sympathetic chain ganglia were incubated with peritoneal fluid, which was demonstrated to repel the nerve fiber outgrowth and the repellence was attempted to be inhibited by blocking Sema3C or 3F with an antibody. This experiment elucidated, that either Sema3C or 3F can be solely responsible for the axon repellence, no axonal collapse occurred, nevertheless axonal outgrowth continued to be inhibited. Therefore, further neurotrophic factors, expressed in the peritoneal fluid, are crucial for nerve fiber repellence and Semas are probably just partly involved.

4.4 Peritoneal fluid and endometriosis-associated factors induce Sema expression

Sema expression has been demonstrated in a wide range of immune cells, e.g. macrophages and activated fibroblasts (Miller, Weidler, Falk, Angele, Schaumburger, Scholmerich and Straub, 2004). Furthermore, studies in neuronal scar tissue or tissue injury have demonstrated Sema expression in the fibroblast component of the developing scar (De Winter, Holtmaat and Verhaagen, 2002, De Winter, et al., 2002, Pasterkamp, Giger, Ruitenberg, Holtmaat, De Wit, De Winter and Verhaagen, 1999). In this study, the expression of Semas could be evidenced in activated fibroblasts in peritoneal tissue of endometriosis patients. Nevertheless, factors inducing Sema expression in fibroblasts remain widely unknown.

A study analyzed the influence of estrogen on Sema3F expression. Therefore, rats received a chronic estrogen treatment; as a result, Sema3F expression was 5-fold magnified in the immature uterus. The Sema3F expression was induced specifically in connective tissue. The major cell types with increased Sema3F expression were fibroblast-like cells and infiltrating eosinophil leukocytes. Furthermore, Nrp2 expression could be localized in nerve terminal in the estrogenized uterus. Sema3F expression could be correlated with sympathetic denervation in the rat uterus and therefore Sema3F was suggested to convert the estrogenized myometrium into an inhospitable region for sympathetic nerve fibers (Richeri, Chalar, Martinez, Greif, Bianchimano and Brauer, 2011). Similarly, in this study Sema expression was found in significantly higher levels in patients with endometriosis and Sema expression could be identified in fibroblasts. Since endometriosis is a highly estrogen-dependent disease, we aimed

to elucidate if estrogen might influence Sema expression in fibroblasts during endometriosis. Therefore, a fibroblast cell line was incubated with different estrogen concentrations and RNA for gene expression analysis was isolated after different time points. Through this, we could demonstrate that Sema3C, 3F and even Nrp2 expression can be modulated through estrogen and might be a crucial factor during endometriosis leading to the induction of Sema and Nrp2 expression.

Furthermore, the influence of TGF on Sema and Nrp expression was tested on fibroblasts. TGF is a highly conserved protein, belonging to the cytokines signaling molecules and is widely known for its role during tissue repair and tissue fibrosis. During wound healing and organ fibrosis, TGF-β mediates fibroblast activation. Furthermore, TGF-β is involved in fibroblast recruitment (Ronnov-Jessen and Petersen, 1993) (Kalluri and Zeisberg, 2006). TGF-β is expressed in the endometrium and secreted be endometrial cells and macrophages. This study and others have demonstrated significantly increased levels of highly active macrophages during endometriosis (Berbic, et al., 2009, Taylor, et al., 1997, Wu and Ho, 2003). Therefore, it is known that TGF-β is secreted into the peritoneal fluid in endometriosis patients. Since it is also responsible for fibroblasts activation, we hypothesized an induction of Sema expression in fibroblasts after treatment with TGF-β. Indeed, Sema3C, 3F and Nrp2 expression was significantly increased in fibroblasts after TGF-β treatment. Suggesting that TGF-β in peritoneal endometriosis might contribute to fibroblast activation and thereby directly or indirectly induce Sema expression.

Treatment of fibroblasts with estrogen and TGF-β induced Sema and Nrp expression. Estrogen and TGF-β are important factors during endometriosis and present in the peritoneal fluid of endometriosis patients. The peritoneal fluid also contains further more important growth factors and cytokines named above, which might also influence fibroblast activation or induction of Sema expression. Therefore, in this study the effects of peritoneal fluid of women with endometriosis were tested in vitro on a fibroblast cell line. The treatment elucidated an increase of Sema3C, 3F and Nrp2 expression in the fibroblasts. Importantly, only the peritoneal fluid of women with endometriosis leaded to the increase of Sema and Nrp expression and not the fluid of women without endometriosis. These results suggest that the peritoneal fluid of women with endometriosis is different from peritoneal fluid of women without endometriosis but most importantly, the peritoneal fluid of women with endometriosis must contain

factors able of inducing Sema expression, which are not present in peritoneal fluid of healthy women.

Fibroblasts are mainly known for their role as producers of extracellular matrix and even if they are the most abundant cells of the stroma, the characterization on molecular terms remains relatively unknown (Chang, Chi, Dudoit, Bondre, van de Rijn, Botstein and Brown, 2002). However, fibroblasts are known to play a role during immune responses by producing cytokines and chemokines for example (Flavell, Hou, Lax, Filer, Salmon and Buckley, 2008). Furthermore, fibroblasts have been suggested to modify the quality, quantity and duration of inflammatory infiltrates (Parsonage, Filer, Haworth, Nash, Rainger, Salmon and Buckley, 2005). Unfortunately, the exact role or activities of fibroblasts during the chronic inflammation in endometriosis remain widely unknown. Nevertheless, the results in this study suggest inflammatory responses of the fibroblasts due to their activity status and contribution to sympathetic nerve fiber repellence by Sema expression.

5 Summary

The widespread phenomenon of a sympathetic hypoinnervation during chronic inflammatory diseases, which was also shown in peritoneal endometriosis, could be verified in this study. The reduction of these anti-inflammatory nerve fibers during endometriosis, occurs in the endometriosis affected tissue, but also in the unaffected tissue of women with endometriosis, suggesting an overall impairment of the peritoneal cavity during peritoneal endometriosis. Our analysis revealed an over expression of Sema 3C and 3F in peritoneal endometriotic tissue. Further, in peritoneal fluid of endometriosis patients, an over expression of Sema 3C and the soluble form of its specific receptor, Nrp 2, was identified. The specific Sema receptors, which are known to be involved in the sympathetic nerve fiber repellence mechanisms, were identified on sympathetic nerve fibers only in peritoneal tissue of women with endometriosis and not without endometriosis. Analysis of Sema and receptors expression in serum of endometriosis patients revealed no significant results, suggesting that endometriosis is a disease of the peritoneal cavity and not systemic.

Interestingly, Sema expression was identified in pivotal cells during inflammation, macrophages and activated fibroblasts. Therefore, Sema interactions during endometriosis are probably regulated by mechanisms of the immune response. Finally, suggesting a neuroimmunomodulation of nerve fibers during the pathogenesis of endometriosis.

Furthermore, it could be shown that peritoneal fluid of women with endometriosis is insofar affected as it is able to cause sympathetic nerve fiber repulsion in vitro, similarly as Semas do, which was verified in this study. Effects of the endometriotic peritoneal fluid on sympathetic ganglia could only be partially inhibited by Semas antibodies, suggesting that further factors expressed in the peritoneal fluid of EM patients, are able to affect sympathetic axon growth.

The expression of Semas and Nrp 2 in fibroblasts, could be regulated in vitro by treatment with endometriosis associated factors such as estrogen and TGF, but more importantly by the treatment with peritoneal fluid of endometriosis patients and not by treatment with peritoneal fluid of women without endometriosis. This result demonstrates clear differences in the properties of peritoneal fluid during

endometriosis or unaffected by the disease. Furthermore, it demonstrates that the peritoneal fluid has a key role during the regulation of Sema expression during endometriosis.

Altogether, this study demonstrates that a repulsion of sympathetic nerve fibers in the peritoneum of women with endometriosis might be regulated by neruoimmunomodulatory mechanisms involving the Sema pathway. Thereby, the anti-inflammatory acting sympathetic nervous system might be impaired and the chronic inflammatory state during endometriosis is promoted.

6 Zusammenfassung

Das weitverbreitete Phänomen einer sympathischen Hypoinnervation in chronisch-entzündlichen Erkrankungen, welches auch in der peritonealen Endometriose nachgewiesen wurde, konnte in dieser Studie bestätigt werden. Dabei zeigt sich nicht nur direkt in den Endometrioseläsionen eine verminderte Anzahl dieser anti-inflammatorischen Nervenfasern, sondern auch im nicht betroffenen Gewebe von Frauen mit Endometriose. Dieses Ergebnis deutet darauf hin, dass die gesamte Peritonealhöhle bei Patientinnen mit peritonealen Endometrioseläsionen betroffen ist. Unsere Analysen zeigten eine erhöhte Expression von Sema 3C und Sema 3F in peritonealem endometriotischem Gewebe. Zusätzlich konnte in der peritonealen Flüssigkeit eine erhöhte Expression von Sema 3C und dem zugehörigen Rezeptor Nrp 2 identifiziert werden. Besonders hervorzuheben ist, dass die spezifische Sema Rezeptoren, die eine wichtige Rolle im Prozess der Modulation sympathischer Nervenfasern spielen, nur auf Endometriose-assoziierten Nervenfasern exprimiert wurden. In Geweben von nicht Endometriosepatientinnen wurden sie hingegen nicht exprimiert.

Die Expressionsanalysen von pro- und antiinflammatorischen Faktoren im Serum ergaben keine signifikanten Veränderungen, was darauf hindeutet, dass die Endometriose überwiegend zu lokalen inflammatorischen Veränderungen führt.

Interessanterweise wurden Semas und Nrp 2 in inflammatorischen Zellen wie Makrophagen und aktivierten Fibroblasten gefunden. Daher liegt es nahe, dass die Sema Regulation durch Endometriose-assoziierte Immunprozesse beeinflusst wird und schlussendlich die Neuroimmunmodulation der Nervenfasern ein wichtiger Schlüsselfaktor in der Pathogenese der Endometriose darstellt.

Die Peritonealflüssigkeit von Patientinnen mit Endometriose zeigt ähnliche Eigenschaften wie die Semas selbst und führt im *in vitro* Versuch zu einer Abstoßung/Schädigung sympathischer Nervenfasern. Die Effekte der endometriotischen Peritonealflüssigkeit auf sympathische Nervenfasern konnte teilweise durch Sema Antikörper inhibiert werden. Dies suggeriert, dass es weitere Nerven abstoßende Faktoren in der Peritonealflüssigkeit bei der Endometriose geben muss, welche diesen Prozess beeinflussen.

Die Expression von Semas und Nrp 2 in Fibroblasten, konnte in *in vitro* Versuchen durch Endometriose-assoziierte Faktoren wie Östrogen und TGF reguliert werden. Wesentlich bedeutender war allerdings die Erkenntnis, dass die Peritonealflüssigkeit von erkrankten Frauen ebenfalls eine regulierende Wirkung zeigte, während die Peritonealflüssigkeit von gesunden Frauen keinen Effekt hatte. Dies zeigt deutlich die unterschiedlichen Eigenschaften der Peritonealflüssgkeiten von Frauen mit Endometriose und Gesunden. Außerdem, weisen diese Ergebnisse auf eine entscheidende Rolle der Peritonealflüssigkeit bei der Regulierung der Sema Expression während der Endometriose Erkrankung hin.

Insgesamt zeigt die Studie, dass die Abstoßung von sympathischen Nervenfasern im Peritoneum von Frauen mit Endometriose durch neuroimmunmodulatorische Mechanismen des Sema Signalweges möglich wäre. Dementsprechend würde dies zu einer Reduktion der anti-inflammatorischen sympatischen Nervenfasern und somit den Weg für den chronischen Inflammationsprozess begünstigen. Weiterführende Untersuchungen könnten hier neue Wege der gezielten anti-inflammatorischen Therapie aufzeigen.

LIST OF FIGURES

References

Adams RH, Lohrum M, Klostermann A, Betz H, Puschel AW. The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. *EMBO J* 1997;16: 6077-6086.
Anaf V, Simon P, El Nakadi I, Fayt I, Simonart T, Buxant F, Noel JC. Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. *Hum Reprod* 2002;17: 1895-1900.
Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* 2001;1: 31-40.
Arnold J, Barcena de Arellano ML, Ruster C, Vercellino GF, Chiantera V, Schneider A, Mechsner S. Imbalance between sympathetic and sensory innervation in peritoneal endometriosis. *Brain Behav Immun* 2012;26: 132-141.
ASRM. Revised American Society for Reproductive Medicine Classification of endometriosis: 1996. *Fertil Steril* 1997;67: 817-821.
Atwal G, du Plessis D, Armstrong G, Slade R, Quinn M. Uterine innervation after hysterectomy for chronic pelvic pain with, and without, endometriosis. *Am J Obstet Gynecol* 2005;193: 1650-1655.
Barcena de Arellano ML, Arnold J, Vercellino F, Chiantera V, Schneider A, Mechsner S. Overexpression of nerve growth factor in peritoneal fluid from women with endometriosis may promote neurite outgrowth in endometriotic lesions. *Fertil Steril* 2011;95: 1123-1126.
Barcena de Arellano ML, Oldeweme J, Arnold J, Schneider A, Mechsner S. Remodeling of estrogen-dependent sympathetic nerve fibers seems to be disturbed in adenomyosis. *Fertil Steril* 2013;100: 801-809.
Batt RE. A History of Endoemtriosis *Springer Verlag*. 2011.
Becker PM, Waltenberger J, Yachechko R, Mirzapoiazova T, Sham JS, Lee CG, Elias JA, Verin AD. Neuropilin-1 regulates vascular endothelial growth factor-mediated endothelial permeability. *Circ Res* 2005;96: 1257-1265.
Beggs JL, Waggener JD. Microvascular regeneration following spinal cord injury: the growth sequence and permeability properties of new vessels. *Advances in neurology* 1979;22: 191-206.
Berbic M, Schulke L, Markham R, Tokushige N, Russell P, Fraser IS. Macrophage expression in endometrium of women with and without endometriosis. *Hum Reprod* 2009;24: 325-332.
Binch ALA, Cole AA, Breakwell LM, Michael ALR, Chiverton N, Creemers LB, Cross AK, Le Maitre CL. *Class 3 semaphorins expression and association with innervation and angiogenesis within the degenerate human intervertebral disc*, 2015.
Borghese B, Vaiman D, Mondon F, Mbaye M, Anaf V, Noel JC, de Ziegler D, Chapron C. [Neurotrophins and pain in endometriosis]. *Gynecol Obstet Fertil* 2010;38: 442-446.
Branton MH, Kopp JB. TGF-beta and fibrosis. *Microbes and infection / Institut Pasteur* 1999;1: 1349-1365.
Brot S, Auger C, Bentata R, Rogemond V, Menigoz S, Chounlamountri N, Girard-Egrot A, Honnorat J, Moradi-Ameli M. Collapsin response mediator protein 5 (CRMP5) induces mitophagy, thereby regulating mitochondrion numbers in dendrites. *J Biol Chem* 2013;289: 2261-2276.
Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 2001;22: 199-204.
Capellino S, Straub RH. Neuroendocrine immune pathways in chronic arthritis. *Best Pract Res Clin Rheumatol* 2008;22: 285-297.
Carvalho L, Podgaec S, Bellodi-Privato M, Falcone T, Abrao MS. Role of eutopic endometrium in pelvic endometriosis. *J Minim Invasive Gynecol* 2011;18: 419-427.
Cerani A, Tetreault N, Menard C, Lapalme E, Patel C, Sitaras N, Beaudoin F, Leboeuf D, De Guire V, Binet F *et al.* Neuron-derived semaphorin 3A is an early inducer of vascular permeability in diabetic retinopathy via neuropilin-1. *Cell metabolism* 2013;18: 505-518.

Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A* 2002;99: 12877-12882.
Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C, Moses MA, Mulligan RC, Folkman J, Kaipainen A. Dose-dependent response of FGF-2 for lymphangiogenesis. *Proc Natl Acad Sci U S A* 2004;101: 11658-11663.
Charnock-Jones DS, Macpherson AM, Archer DF, Leslie S, Makkink WK, Sharkey AM, Smith SK. The effect of progestins on vascular endothelial growth factor, oestrogen receptor and progesterone receptor immunoreactivity and endothelial cell density in human endometrium. *Hum Reprod* 2000;15 Suppl 3: 85-95.
Chedotal A, Del Rio JA, Ruiz M, He Z, Borrell V, de Castro F, Ezan F, Goodman CS, Tessier-Lavigne M, Sotelo C *et al.* Semaphorins III and IV repel hippocampal axons via two distinct receptors. *Development* 1998;125: 4313-4323.
Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 1997;19: 547-559.
Cheng HJ, Bagri A, Yaron A, Stein E, Pleasure SJ, Tessier-Lavigne M. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. *Neuron* 2001;32: 249-263.
Chishima F, Hayakawa S, Yamamoto T, Sugitani M, Karasaki-Suzuki M, Sugita K, Nemoto N. Expression of inducible microsomal prostaglandin E synthase in local lesions of endometriosis patients. *Am J Reprod Immunol* 2007;57: 218-226.
Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ. Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 1996;271: 736-741.
Crosignani PG, Vercellini P, Biffignandi F, Costantini W, Cortesi I, Imparato E. Laparoscopy versus laparotomy in conservative surgical treatment for severe endometriosis. *Fertil Steril* 1996;66: 706-711.
De Winter F, Holtmaat AJ, Verhaagen J. Neuropilin and class 3 semaphorins in nervous system regeneration. *Adv Exp Med Biol* 2002;515: 115-139.
De Winter F, Oudega M, Lankhorst AJ, Hamers FP, Blits B, Ruitenberg MJ, Pasterkamp RJ, Gispen WH, Verhaagen J. Injury-induced class 3 semaphorin expression in the rat spinal cord. *Exp Neurol* 2002;175: 61-75.
Demir Weusten AY, Groothuis PG, Dunselman GA, de Goeij AF, Arends JW, Evers JL. Morphological changes in mesothelial cells induced by shed menstrual endometrium in vitro are not primarily due to apoptosis or necrosis. *Hum Reprod* 2000;15: 1462-1468.
Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. *Hum Reprod* 1998;13: 1686-1690.
Drake TS, O'Brien WF, Ramwell PW, Metz SA. Peritoneal fluid thromboxane B2 and 6-keto-prostaglandin F1 alpha in endometriosis. *Am J Obstet Gynecol* 1981;140: 401-404.
Drosdzol-Cop A, Skrzypulec-Plinta V, Stojko R. Serum and peritoneal fluid immunological markers in adolescent girls with chronic pelvic pain. *Obstet Gynecol Surv* 2012;67: 374-381.
Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* 2000;52: 595-638.
Evans S, McShane P. The efficacy of internal iliac artery ligation in obstetric hemorrhage. *Surg Gynecol Obstet* 1985;160: 250-253.
Fassold A, Falk W, Anders S, Hirsch T, Mirsky VM, Straub RH. Soluble neuropilin-2, a nerve repellent receptor, is increased in rheumatoid arthritis synovium and aggravates sympathetic fiber repulsion and arthritis. *Arthritis Rheum* 2009;60: 2892-2901.
Ferrara N. VEGF-A: a critical regulator of blood vessel growth. *European cytokine network* 2009;20: 158-163.
Ferrero S, Haas S, Remorgida V, Camerini G, Fulcheri E, Ragni N, Straub RH, Capellino S. Loss of sympathetic nerve fibers in intestinal endometriosis. *Fertil Steril* 2010;94: 2817-2819.

Flavell SJ, Hou TZ, Lax S, Filer AD, Salmon M, Buckley CD. Fibroblasts as novel therapeutic targets in chronic inflammation. *Br J Pharmacol* 2008;153 Suppl 1: S241-246.
Flierl MA, Rittirsch D, Nadeau BA, Chen AJ, Sarma JV, Zetoune FS, McGuire SR, List RP, Day DE, Hoesel LM *et al.* Phagocyte-derived catecholamines enhance acute inflammatory injury. *Nature* 2007;449: 721-725.
Gazvani R, Templeton A. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction* 2002;123: 217-226.
Giger RJ, Pasterkamp RJ, Holtmaat AJ, Verhaagen J. Semaphorin III: role in neuronal development and structural plasticity. *Prog Brain Res* 1998;117: 133-149.
Giudice LC. Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. *Fertil Steril* 1994;61: 1-17.
Giudice LC, Evers JLH, Healy DL. *Endometriosis: Science and Practice*, 2012. Wiley.
Graf N, McLean M, Capellino S, Scholmerich J, Murray GI, El-Omar EM, Straub RH. Loss of sensory and noradrenergic innervation in benign colorectal adenomatous polyps--a putative role of semaphorins 3F and 3A. *Neurogastroenterol Motil* 2012;24: 120-128, e183.
Grando SA, Pittelkow MR, Schallreuter KU. Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. *The Journal of investigative dermatology* 2006;126: 1948-1965.
Groothuis PG, Nap AW, Winterhager E, Grummer R. Vascular development in endometriosis. *Angiogenesis* 2005;8: 147-156.
Gu C, Rodriguez ER, Reimert DV, Shu T, Fritzsch B, Richards LJ, Kolodkin AL, Ginty DD. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 2003;5: 45-57.
Haas S, Capellino S, Phan NQ, Bohm M, Luger TA, Straub RH, Stander S. Low density of sympathetic nerve fibers relative to substance P-positive nerve fibers in lesional skin of chronic pruritus and prurigo nodularis. *J Dermatol Sci* 2010;58: 193-197.
Hever A, Roth RB, Hevezi P, Marin ME, Acosta JA, Acosta H, Rojas J, Herrera R, Grigoriadis D, White E *et al.* Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator. *Proc Natl Acad Sci U S A* 2007;104: 12451-12456.
Ho HN, Wu MY, Chao KH, Chen CD, Chen SU, Yang YS. Peritoneal interleukin-10 increases with decrease in activated CD4+ T lymphocytes in women with endometriosis. *Hum Reprod* 1997;12: 2528-2533.
Imperato-Kalmar EL, McKinney RA, Schnell L, Rubin BP, Schwab ME. Local changes in vascular architecture following partial spinal cord lesion in the rat. *Exp Neurol* 1997;145: 322-328.
Jaffe RB. Importance of angiogenesis in reproductive physiology. *Seminars in perinatology* 2000;24: 79-81.
Ji JD, Park-Min KH, Ivashkiv LB. Expression and function of semaphorin 3A and its receptors in human monocyte-derived macrophages. *Hum Immunol* 2009;70: 211-217.
Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-beta superfamily expression and actions in the endometrium and placenta. *Reproduction* 2006;132: 217-232.
Kaiser A, Kopf A, Gericke C, Bartley J, Mechsner S. The influence of peritoneal endometriotic lesions on the generation of endometriosis-related pain and pain reduction after surgical excision. *Arch Gynecol Obstet* 2009;280: 369-373.
Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006;6: 392-401.
Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H. A requirement for neuropilin-1 in embryonic vessel formation. *Development* 1999;126: 4895-4902.
Keenan JA, Chen TT, Chadwell NL, Torry DS, Caudle MR. Interferon-gamma (IFN-gamma) and interleukin-6 (IL-6) in peritoneal fluid and macrophage-conditioned media of women with endometriosis. *Am J Reprod Immunol* 1994;32: 180-183.
Kennedy MK, Picha KS, Shanebeck KD, Anderson DM, Grabstein KH. Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0, clones. *Eur J Immunol* 1994;24: 2271-2278.
Kiguchi N, Kobayashi Y, Saika F, Sakaguchi H, Maeda T, Kishioka S. Peripheral interleukin-4 ameliorates inflammatory macrophage-dependent neuropathic pain. *Pain* 2015;156: 684-693.

Kikutani H, Suzuki K, Kumanogoh A. Immune semaphorins: increasing members and their diverse roles. *Advances in immunology* 2007;93: 121-143.
Kingery WS. Role of neuropeptide, cytokine, and growth factor signaling in complex regional pain syndrome. *Pain medicine* 2010;11: 1239-1250.
Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H. Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 1995;121: 4309-4318.
Klatt S, Fassold A, Straub RH. Sympathetic nerve fiber repulsion: testing norepinephrine, dopamine, and 17beta-estradiol in a primary murine sympathetic neurite outgrowth assay. *Ann N Y Acad Sci* 2012;1261: 26-33.
Koks CA, Groothuis PG, Dunselman GA, de Goeij AF, Evers JL. Adhesion of shed menstrual tissue in an in-vitro model using amnion and peritoneum: a light and electron microscopic study. *Hum Reprod* 1999;14: 816-822.
Kolodkin AL. Growth cones and the cues that repel them. *Trends Neurosci* 1996;19: 507-513.
Koopman FA, Stoof SP, Straub RH, Van Maanen MA, Vervoordeldonk MJ, Tak PP. Restoring the balance of the autonomic nervous system as an innovative approach to the treatment of rheumatoid arthritis. *Molecular medicine* 2011;17: 937-948.
Lakhan SE, Kirchgessner A. Neuroinflammation in inflammatory bowel disease. *J Neuroinflammation* 2010;7: 37.
Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. *Fertil Steril* 2001;75: 1-10.
Lech M, Anders HJ. Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochimica et biophysica acta* 2013;1832: 989-997.
Lehner B, Koeck FX, Capellino S, Schubert TE, Hofbauer R, Straub RH. Preponderance of sensory versus sympathetic nerve fibers and increased cellularity in the infrapatellar fat pad in anterior knee pain patients after primary arthroplasty. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2008;26: 342-350.
Levine JD, Coderre TJ, Helms C, Basbaum AI. Beta 2-adrenergic mechanisms in experimental arthritis. *Proc Natl Acad Sci U S A* 1988;85: 4553-4556.
Levine JD, Goetzl EJ, Basbaum AI. Contribution of the nervous system to the pathophysiology of rheumatoid arthritis and other polyarthritides. *Rheum Dis Clin North Am* 1987;13: 369-383.
Leyendecker G, Herbertz M, Kunz G, Mall G. Endometriosis results from the dislocation of basal endometrium. *Hum Reprod* 2002;17: 2725-2736.
Leyendecker G, Kunz G, Noe M, Herbertz M, Mall G. Endometriosis: a dysfunction and disease of the archimetra. *Hum Reprod Update* 1998;4: 752-762.
McCorry LK. Physiology of the autonomic nervous system. *American journal of pharmaceutical education* 2007;71: 78.
McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. *J Clin Invest* 1996;98: 482-489.
McLaren J, Prentice A, Charnock-Jones DS, Smith SK. Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum Reprod* 1996;11: 220-223.
Mechsner S, Schwarz J, Thode J, Loddenkemper C, Salomon DS, Ebert AD. Growth-associated protein 43-positive sensory nerve fibers accompanied by immature vessels are located in or near peritoneal endometriotic lesions. *Fertil Steril* 2007;88: 581-587.
Medicine ASfR. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997;67: 817-821.
Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* 1995;14: 949-959.
Meyer R. Über den Stand der Frage der Adenomyositis und Adenome im allgemeinen und insbesondere über Adenomyositis seroepitheliaslis und Adenomyometritis sarcomatosa. *Gynäkologie* 1919: 745-750.

Miller LE, Justen HP, Scholmerich J, Straub RH. The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages. *Faseb J* 2000;14: 2097-2107.
Miller LE, Weidler C, Falk W, Angele P, Schaumburger J, Scholmerich J, Straub RH. Increased prevalence of semaphorin 3C, a repellent of sympathetic nerve fibers, in the synovial tissue of patients with rheumatoid arthritis. *Arthritis Rheum* 2004;50: 1156-1163.
Montagna P, Capellino S, Villaggio B, Remorgida V, Ragni N, Cutolo M, Ferrero S. Peritoneal fluid macrophages in endometriosis: correlation between the expression of estrogen receptors and inflammation. *Fertil Steril* 2008;90: 156-164.
Montesano R, Orci L. Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. *Proc Natl Acad Sci U S A* 1988;85: 4894-4897.
Mueller MD, Lebovic DI, Garrett E, Taylor RN. Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. *Fertil Steril* 2000;74: 107-112.
Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN. Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. *Proc Natl Acad Sci U S A* 2000;97: 10972-10977.
Nagy JA, Dvorak AM, Dvorak HF. VEGF-A and the induction of pathological angiogenesis. *Annu Rev Pathol* 2007;2: 251-275.
Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987-2007). *Brain Behav Immun* 2007;21: 736-745.
Nap AG, AW; Dunselman, GA; Bouma-Ter Steege, JC; Thijssen, VL; Evers, JL; Groothius, PG. Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab* 2004;89: 1089-1095.
Nap AW. Theories on the Pathogenesis of Endometriosis *Endometriosis*. 2012. Wiley-Blackwell, pp. 42-53.
Nothnick WB. Treating endometriosis as an autoimmune disease. *Fertil Steril* 2001;76: 223-231.
Oliker AJ, Harris AE. Endometriosis of the bladder in a male patient. *J Urol* 1971;106: 858-859.
Omwandho CO, Konrad L, Halis G, Oehmke F, Tinneberg HR. Role of TGF-betas in normal human endometrium and endometriosis. *Hum Reprod* 2010;25: 101-109.
Ozer H, Boztosun A, Acmaz G, Atilgan R, Akkar OB, Kosar MI. The efficacy of bevacizumab, sorafenib, and retinoic acid on rat endometriosis model. *Reprod Sci* 2013;20: 26-32.
Park JS, Lee JH, Kim M, Chang HJ, Hwang KJ, Chang KH. Endometrium from women with endometriosis shows increased proliferation activity. *Fertil Steril* 2009;92: 1246-1249.
Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE, Salmon M, Buckley CD. A stromal address code defined by fibroblasts. *Trends Immunol* 2005;26: 150-156.
Pasterkamp RJ, Giger RJ, Ruitenberg MJ, Holtmaat AJ, De Wit J, De Winter F, Verhaagen J. Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Mol Cell Neurosci* 1999;13: 143-166.
Pinkert TC, Catlow CE, Straus R. Endometriosis of the urinary bladder in a man with prostatic carcinoma. *Cancer* 1979;43: 1562-1567.
Pongratz G, Straub RH. The B cell, arthritis, and the sympathetic nervous system. *Brain Behav Immun* 2010;24: 186-192.
Rana N, Braun DP, House R, Gebel H, Rotman C, Dmowski WP. Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. *Fertil Steril* 1996;65: 925-930.
Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. *J Clin Invest* 2008;118: 3522-3530.
Ricci AG, Olivares CN, Bilotas MA, Meresman GF, Baranao RI. Effect of vascular endothelial growth factor inhibition on endometrial implant development in a murine model of endometriosis. *Reprod Sci* 2011;18: 614-622.
Richeri A, Chalar C, Martinez G, Greif G, Bianchimano P, Brauer MM. Estrogen up-regulation of semaphorin 3F correlates with sympathetic denervation of the rat uterus. *Auton Neurosci* 2011;164: 43-50.

Riepl B, Grassel S, Wiest R, Fleck M, Straub RH. Tumor necrosis factor and norepinephrine lower the levels of human neutrophil peptides 1-3 secretion by mixed synovial tissue cultures in osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther* 2010;12: R110.
Rocha AL, Reis FM, Taylor RN. Angiogenesis and endometriosis. *Obstetrics and gynecology international* 2013;2013: 859619.
Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Laboratory investigation; a journal of technical methods and pathology* 1993;68: 696-707.
Ruediger T, Zimmer G, Barchmann S, Castellani V, Bagnard D, Bolz J. Integration of opposing semaphorin guidance cues in cortical axons. *Cerebral cortex* 2013;23: 604-614.
Sampson J. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927;14: 422-469.
Schindler AE. Operative und medikamentöse Therapie der Endometriose/Adenomyose. *J Gynäkol Endokrinol* 2008;18: 18-26.
Schrodt GR, Alcorn MO, Ibanez J. Endometriosis of the male urinary system: a case report. *J Urol* 1980;124: 722-723.
Schweppe K. Endometriose - Eine Erkrankung ohne Lobby. *Zentralbl Gynäkol* 2003: 233

Sharma A, Verhaagen J, Harvey AR. Receptor complexes for each of the Class 3 Semaphorins. *Front Cell Neurosci* 2012;6: 28.
Sharpe-Timms KL. Endometrial anomalies in women with endometriosis. *Ann N Y Acad Sci* 2001;943: 131-147.
Sharpe-Timms KL. Haptoglobin expression by shed endometrial tissue fragments found in peritoneal fluid. *Fertil Steril* 2005;84: 22-30.
Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* 1996;81: 3112-3118.
Siegel GJ, Agranoff BW. *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 1999. Lippincott Williams & Wilkins.
Sinaii N, Cleary SD, Ballweg ML, Nieman LK, Stratton P. High rates of autoimmune and endocrine disorders, fibromyalgia, chronic fatigue syndrome and atopic diseases among women with endometriosis: a survey analysis. *Hum Reprod* 2002;17: 2715-2724.
Spengler RN, Allen RM, Remick DG, Strieter RM, Kunkel SL. Stimulation of alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. *J Immunol* 1990;145: 1430-1434.
Starzinski-Powitz A, Handrow-Metzmacher H, Kotzian S. The putative role of cell adhesion molecules in endometriosis: can we learn from tumour metastasis? *Molecular medicine today* 1999;5: 304-309.
Stratton P, Berkley KJ. Chronic pelvic pain and endometriosis: translational evidence of the relationship and implications. *Hum Reprod Update* 2011;17: 327-346.
Straub RH. Autoimmune disease and innervation. *Brain Behav Immun* 2007;21: 528-534.
Straub RH, Grum F, Strauch U, Capellino S, Bataille F, Bleich A, Falk W, Scholmerich J, Obermeier F. Anti-inflammatory role of sympathetic nerves in chronic intestinal inflammation. *Gut* 2008;57: 911-921.
Straub RH, Harle P. Sympathetic neurotransmitters in joint inflammation. *Rheum Dis Clin North Am* 2005;31: 43-59, viii.
Suto F, Ito K, Uemura M, Shimizu M, Shinkawa Y, Sanbo M, Shinoda T, Tsuboi M, Takashima S, Yagi T *et al.* Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. *J Neurosci* 2005;25: 3628-3637.
Takahashi T, Nakamura F, Jin Z, Kalb RG, Strittmatter SM. Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. *Nat Neurosci* 1998;1: 487-493.
Taylor RL, DI; Mueller MD. Angiogenic factors in endometriosis. *NY Acad Sci* 2002;955: 89-100.

Taylor RN, Ryan IP, Moore ES, Hornung D, Shifren JL, Tseng JF. Angiogenesis and macrophage activation in endometriosis. *Ann N Y Acad Sci* 1997;828: 194-207.
Tran LV, Tokushige N, Berbic M, Markham R, Fraser IS. Macrophages and nerve fibres in peritoneal endometriosis. *Hum Reprod* 2009;24: 835-841.
Waimey KE, Huang PH, Chen M, Cheng HJ. Plexin-A3 and plexin-A4 restrict the migration of sympathetic neurons but not their neural crest precursors. *Dev Biol* 2008;315: 448-458.
Wang G, Tokushige N, Markham R, Fraser IS. Rich innervation of deep infiltrating endometriosis. *Hum Reprod* 2009;24: 827-834.
Weidler C, Holzer C, Harbuz M, Hofbauer R, Angele P, Scholmerich J, Straub RH. Low density of sympathetic nerve fibres and increased density of brain derived neurotrophic factor positive cells in RA synovium. *Ann Rheum Dis* 2005;64: 13-20.
Wu MY, Ho HN. The role of cytokines in endometriosis. *Am J Reprod Immunol* 2003;49: 285-296.
Wu MY, Ho HN, Chen SU, Chao KH, Chen CD, Yang YS. Increase in the production of interleukin-6, interleukin-10, and interleukin-12 by lipopolysaccharide-stimulated peritoneal macrophages from women with endometriosis. *Am J Reprod Immunol* 1999;41: 106-111.
Yaron A, Huang PH, Cheng HJ, Tessier-Lavigne M. Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins. *Neuron* 2005;45: 513-523.