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„High resolution mass spectrometry based Molecular profiling of endometriosis epithelial cell line 12-Z and endometrial cell line THESC upon simulated macrophage derived inflammatory signaling reveals neurogenic activity of endometriotic cells"

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Zusammenfassung:

Endometriose, eine östrogenabhängige chronisch entzündliche gynäkologische Erkrankung, betrifft Millionen von Frauen weltweit und beeinträchtigt ihre Lebensqualität erheblich und verursacht aufgrund chronischer neurogener Entzündungen enorme Schmerzen. Obwohl eine von zehn Frauen an Endometriose leidet, bleibt die Behandlung eine Herausforderung. Verschiedene pathogene Theorien wurden vorgeschlagen, aber der Ursprung der Endometriose bleibt unbekannt. Eine erhöhte entzündliche Zytokinproduktion von TNFα und Il1β, die durch Endometriose -assoziierte Makrophagen sekretiert wird, wurde als wichtiger Faktor bei dem Fortschreiten der Krankheit beschrieben. Die molekulare Funktion dieser entzündlichen Zytokine hinsichtlich der Endometriose -Pathogenese sowie die molekularen Merkmale, die die Wechselwirkung zwischen ektopischen Läsionen und der umgebenden Mikroumgebung definieren, sind noch unklar.Mit der Entwicklung von Omics-Techniken wie Proteomik stehen leistungsstarke Werkzeuge zur Verfügung, um ganzheitlichere Untersuchungen unbekannter Pathomechanismen zu ermöglichen und deren Verständnis zu fördern. Hier wurde ein massenspektrometrisches Bottom-Up-Proteomics-Profiling verwendet, um ein tieferes Verständnis der zugrunde liegenden molekularen Mechanismen während der von Makrophagen abgeleiteten Entzündungssignale in endometriotischen Läsionen zu schaffen. Zwei verschiedene Zelllinien, nämlich die Endometriose 12-Z-Epithelzelllinie und die endometriale Stroma -Fibroblasten-Zelllinie THESC, wurden mit IL1β und TNFα behandelt, um zwei kanonische entzündliche Signaltransduktionskaskaden zu simulieren, die für die Beteiligung von Makrophagen charakteristisch sind. Die stimulierten Zellen wurden mit nicht behandelten verglichen, wobei regulierte Proteine für eine auf Genontologie basierende Pathway-Netzwerkanalyse verwendet worden sind.Der Vergleich der Endometriose-Zelllinie 12-Z und der Nicht-Endometriose-Zelllinie THESC ergab bereits tiefgreifende Unterschiede, insbesondere charakteristische Eigenschaften von Endometriose- Zellen. Beide Zelllinien zeigten bei Stimulation eine Entzündungsreaktion. Weiters ergab die Proteomanalyse einige Unterschiede zwischen der Reaktion der entzündlich aktivierten Endometriosezellinie im Vergleich zur Stromazellantwort. Interessanterweise, wurden zusätzlich zur kanonischen Entzündungsreaktion mit IL1β und TNFα verschiedene pathologische Merkmale beobachtet. Dementsprechend wurden Unterschiede zwischen der Nicht-Endometriose-Zelllinie THESC und der Endometriose-Zelllinie 12-Z festgestellt, was auf spezielle Endometriose typische pathologische Eigenschaften hinweist. Nur die endometriotische 12-Z Zellinie ergab bei der Analyse eine aberrante Regulation der Neurogenese, Epitheliale Mesenchymale Transition, Angiogenese und der Aktivierung der embryonalen Entwicklungstransduktion. Ebenso wurden entscheidende Proteine, welche wesentlich für die Semaphorin Signaltransduktion sind, bei Stimulation mit TNFα erhöht vorgefunden. Daher lässt sich daraus schließen, dass die von Makrophagen abgeleitete entzündliche Tumornekrosefaktor-α-Stimulation die Aktivierung der axonalen Wegfindung von endometriotischen Epithelzellen zur Folge hat. Im Gegensatz dazu zeigte die THESC-Zelllinie biologische Funktionen auf, die an der Implantation beteiligt sind. Ebenso wurden Proteine die an zellnervösen Interaktionen beteiligt sind, im Gegensatz zur Endometriose Zelllinie, durch die vorangegangene entzündliche Behandlung in verringter Anzahl vorgefunden. Hier beschreiben wir eine bisher unbekannte direkte Aktivierung der axonalen Leitsignale über das proinflammatorische Zytokin TNFα in einem Endometriose-Zellkulturmodell. Weitere Forschungen sind erforderlich, um die Wechselwirkungen zwischen Neuronen und Zellen unter Makrophagenstimulation zu untersuchen und diese Befunde in vivo zu validieren. Hormonelle und eikosadomische Analyse der Wechselwirkung zwischen Makrophagen und primären Material könnte tiefere Einblicke in Endometriose spezifische neurogene Merkmale geben.

Abstract:

Endometriosis, an estrogen-dependent chronic inflammatory gynecological disease, is affecting millions of women worldwide and profoundly effects their life quality and causes enormous pain due to chronic neurogenic inflammation. Although one in 10 women is suffering from endometriosis, treatment still remains a challenge. Various pathogenic theories have been proposed but the origin of endometriosis remains unknown and requires closer investigation. Increased inflammatory cytokine production of TNFα and Il1β, secreted by endometriosis associated macrophages have been described as important key players in the establishment and progression of the disease. The molecular function of these inflammatory cytokines concerning endometriosis pathogenesis, as well as the molecular features that define the interaction between ectopic lesions and the surrounding microenvironment is still unclear.

With the development of omics techniques like proteomics powerful tools are available to enable more holistic investigations of complex systems and promote their understanding. Here, a mass spectrometry-based bottom-up proteomics profiling was used. To create a deeper understanding of the underlying molecular mechanisms during macrophage derived inflammatory signaling in endometriotic lesions a cell culture model was employed. Two different cell lines, namely the 12-Z endometriotic epithelial cell line and the non-endometriosis endometrial stromal fibroblast cell line THESC were treated with IL1β and TNFα to simulate two canonical inflammatory signaling pathways characteristic for macrophage involvement. The treatments were compared to non-treated cell lines and the regulated proteins were used for gene ontology based pathway network analysis.

The comparison of the endometriosis cell line 12-Z and non-endometriosis cell line THESC already revealed profound differences especially characteristic properties of endometriosis derived cells. Both cell lines exhibited inflammatory response signature upon stimulation. Proteomic analysis disclosed various differences between endometriosis behavior featuring inflammatory activation in comparison to non-endometriosis stromal cell response. Most interestingly, additional to canonical inflammatory response various pathological features were observed upon stimulation with IL1β and TNFα. Accordingly, differences between non-endometriosis cell line THESC and endometriosis cell line 12-Z were detected, indicating special endometriosis linked pathologic qualities. The analysis revealed aberrant regulation of neurogenesis, epithelial-to-mesenchymal transition, angiogenesis and embryonic developmental signaling activation in 12-Z endometriotic cell line, only. Ultimately, crucial proteins involved in processes essential for semaphorin signaling were found to be increased upon stimulation with TNFα. Thus, indicating axonal guidance activation of endometriotic epithelial cells upon macrophage derived inflammatory Tumor necrosis-factor α stimulation. In contrary, THESC cell line featured biological function involved in implantation as well as downregulation of proteins essential for cell-nervous interactions.

Here, we describe a so far unknown direct activation of axonal guidance signaling via the pro-inflammatory cytokine TNFα in an endometriosis cell culture model. Further research is needed to investigate neuron-cell interactions upon macrophage stimulation and validate the findings in vivo. Primary cell culture and derived hormonal and eicosadomic analysis of macrophage –cell interaction could give further insight to specific neurogenic features.

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1. Endometriosis

Endometriosis is a chronic inflammatory, estrogen dependent disease. The depilating gynecological condition, describes abnormal growth of endometrial glands and stroma, which are normally located in the inner lining of the uterus, outside the uterine cavity. The endometrial tissue can therefore grow primarily in the pelvic area and adhere to the ovaries, fallopian tubes, peritoneum, gastrointestinal tract, bladder and recto-vaginal septum. However it is not limited to the pelvic area, endometriosis has also been found in the pericardium and pleura(*1, 2*).

1.1 Classification:

Endometriosis was first classified by Sampson in 1921, when he classified ovarian hemorrhagic cysts. He divided them into endometrial, stromal , corpus luteal and follicular, based on their histological appearance (*1*). Nowadays, there are various classification systems such as ASRM.

According to the American society for reproductive medicine (ASRM), endometriosis can be classified into 4 stages: minimal stage I, mild stage II, moderate stage III or severe stage IV. The stage describes the extent of the abnormal tissue growth, but not the pain the patient is having. The extent of the disease does not correlate with pain or discomfort of the patient (*2–4*). The ASRM staging as well as Enzian classification describe endometriosis according to its depth or whether it is superficial, whether the posterior cul-de-sac is partially or completely obliterated and whether the adhesions, which are often located around ovaries and fallopian tubes are dense ore wide. Also in ASRM the ectopic lesions are described by their color(*5, 6, 2*). There are red, red-pink, clear, white, yellow-brown , black or blue lesions (*2*). Clear and white lesions, or occult endometriosis is very hard to detect and often missed during laparoscopy(*7, 8*).

1.2 Symptoms:

One in 10 women around the world suffers from endometriosis. It is estimated that 70 million woman are affected (*2*). Not every woman experiences the same symptoms. Some doesn't have pain at all. However, the main symptoms are pelvic pain, dysmenorrhea, dyspareunia, dysuria, dyschezia and gastrointestinal problems and discomfort. Painful bowel movements and cyclical leg pain also occur at higher percentage in woman with endometriosis. (*9, 2, 10*) Endometriotic lesions infiltrate and compress the nerves surrounding them. Also they build up their own nerves, which are stimulated by inflammatory sensory nerve fibers, which sense and also transmit pain stimuli to the CNS , because of their afferent fun

ction. (*2*) Some nerves release substance P, calcitonin, gene-related peptide, tachykinins and nitric oxide, which ultimately leads to neurogenic inflammation and also increases local vascular permeability, which enhances migration of ectopic endometrial cells. (*2, 11–13*) One of the main symptoms woman with endometriosis suffer from is infertility. (*14*) The average time to diagnose a woman with endometriosis takes about 7-8 years. The patient is often misdiagnosed with pelvic inflammatory disease, irritable bowel syndrome or other diseases. (*2*)

1.3 Theories:

1.3.1 Sampson's Theory

Sampson's Theory of Endometriosis was the first ever hypothesized in 1927. In his theory Sampson states, that the cause of endometriosis is retrograde menstruation, thus a presence of endometrial cell outside the uterine cavity, which are able to implant themselves and grow to form peritoneal lesions. (*15*) Retrograde menstruation means the regression of menstrual blood, including apoptotic endometrial cells, erythrocytes, macrophages and other menstrual remnants, through the fallopian tube into the peritoneal cavity. The cells then attach to local tissue and form their own nerve endings and start angiogenesis to build up their own blood supply. However, retrograde menstruation occurs in most woman, normally the immune system is preventing endometriosis (*16*)(Rolla 2019)(Rolla 2019)(Rolla 2019)(*16*)(Rolla 2019)(*16*)(Rolla 2019)(*16*) by destroying the lesions, before they can even start angiogenesis. (*2, 16, 17*) The endometrial cells outside the uterine lining are very viable and grow extremely fast, they already have integrins on their cell surface to attach to the peritoneum. (*18, 19, 2*)

1.3.2 The coelomic metaplasia:

Another theory states, that normal cell derivates of primitive peritoneum transform themselves into endometrial tissue. (*20*) The metaplasia theory gives a possible link to woman, who suffer from endometriosis who had a hysterectomy or are post-menopausal. Some evidence even showed ectopic endometrial cells in human fetuses. (*2, 21*) The theory states that ovaries and Mullerian ducts are derived from cells of the coelomic epithelium. The epithelial cells transform and build up tissue like the endometrium. Because the coelomic epithelium is a common ancestor to both cell types, it could transform into the other cell type triggered by inflammation. The metaplasia theory is not proven and might not be correct. If so endometriosis would be present in healthy males or woman born without a uterus. Also, endometriosis would be found at various locations in the body, where tissue derived from coelomic peritoneum. (*22, 23*)

1.3.3 Embryonic Rest Theory:

The theory states that by the presence of cells derived from Mullerian cells, endometrial tissue can be formed. Endometriosis is often present in the recto-vaginal septum as well as along the migration pathway of the embryonic Mullerian system. This theory is not yet proven and remains hypothetical. (*21*)

1.3.4 Lymphatic and Vascular Metastasis:

In retrograde menstruation theory endometrial cells are spreading through the fallopian tube. In this theory endometrial tissue is transported through the vasculature and lymph nodes. The theory is very likely, due to the fact that endometriosis most of the time infiltrates the ovaries. Also, endometriosis is present in more distal locations, such as the pleura or pericardium. Lymphatic and hematogenous transport is very likely. Also, Sampson states that fragments of endometrial tissue might travel through the lymphatic circulation to attach to ectopic sites in order to implant there. (*15*) Accordingly, endometriosis is also present in lymphatic vessels and lymph nodes. (*24, 25*) Throughout the endometrium and also the myometrium lymphatic vessels are distributed.

During menstruation density of endometrial lymphatic vessels is highest, which would support Sampson's theory.(*26*) Uterine lymph angiogenesis is induced by vascular endothelial growth factor C (VEGF-C) and VEGF-D. In woman with endometriosis the expression of VEGFC and –D is altered. It lacks the cyclic variations, also the genexpression of VEGF-C is decreased, while the expression of VEGF-D is significantly higher in endometriosis patients in the secretory phase. Molecules usually produced in the uterus to regulate expression of VEGF are inhibited in their function, while inflammatory cytokines like interleukin 1b (IL1b) and tumor necrosis factor a (TNFα) upregulate the expression of VEGF-C.(*26*) The lymphatic theory would also be likely , to explain adenomyosis, which is endometriosis within the myometrium of the uterus. (*27–29*)

1.3.5 TIAR – Tissue Injury and Repair Theory

TIAR is a relatively new theory, which postulates that the cause of endometriosis is trauma. Chronic peristalsis or a state of hyperperistalsis leads to microtraumatizations. The auto-traumatization, induced by a positive feedback of estrogen stimulation, leads to a high motility of endometrial cells. Thus, it allows the endometrial cells to implant in ectopic tissue. The local estrogen production is increased by tissue injury and repair. The TIAR theory also plays an important role in adenomyosis. (*2, 28*) The thesis is based on the constant activity of the non-pregnant uterus throughout the lifetime of a woman. The molecular mechanism of the mechanical strain involves the local production of estrogen and P450 aromatase. Tissue injury and repair in utero follows a specific system, first estradiol exerts its healing effects via the estrogen receptor b (ER2) therefore, estrogen dependent genes are upregulated. Usually, after tissue has been injured, healing leads to an inflammatory process that involves cell proliferation and the sprouting of sensory nerve fibers. After the healing, the inflammatory process is stopped and all of TIAR is terminated, which includes the inactivation of estrogen production and nerve fibers regressing again (*30*). Autotraumatization caused by hyperperistalsis is induced by the local production of estrogen. This results in an increased desquamation of fragments of the basal endometrium. In combination with increased retrograde uterine transport according to Sampson's theory there is an enhanced trans tubal dissemination of these fragments. For microtraumatization even few increments in

mechanical strain result in activation of COX-2 and therefore in the production of PGE2 and the production of IL-8, which is the most basic biochemical process when it comes to tissue. Accordingly, the repeated and constant overstretching and therefore injury of the myocytes and fibroblasts at the endometrial-myometrial interface lead to activation of the TIAR and an increased production of estradiol.(*30*) In Figure one the basic mechanism of TIAR is shown.

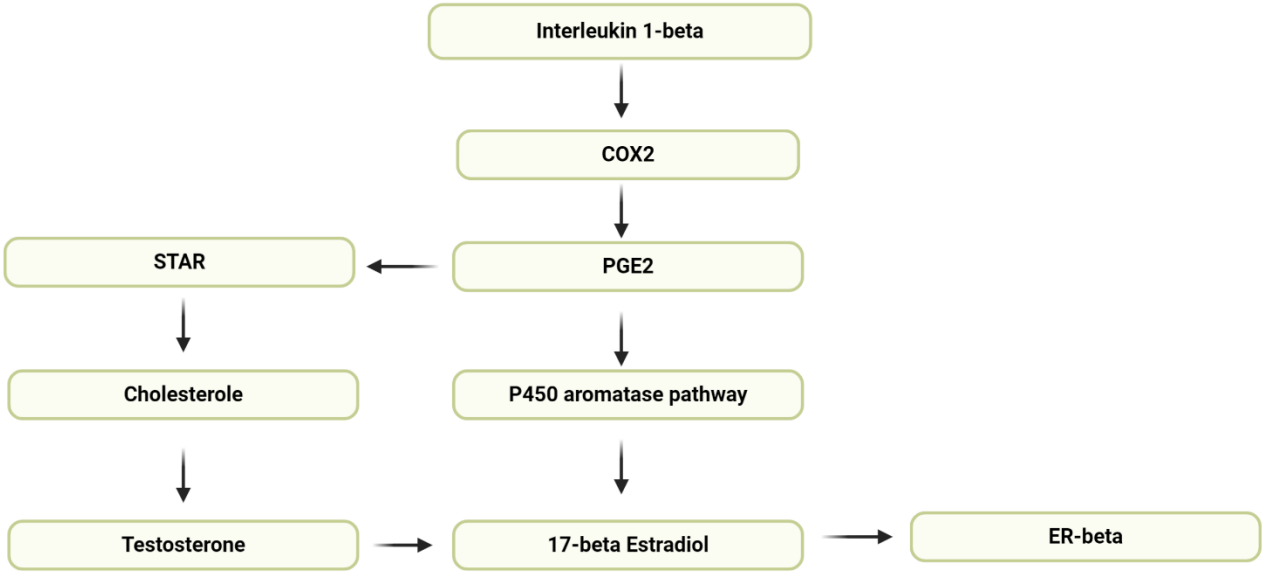


Figure 1 TIAR mechanism modified from (*30*)

The extension of injury and constant hyperperistalsis leads to paracrine estrogen effects and the inflammatory process of TIAR is reinitiated. Thus, resulting in the perpetuating proliferation of stromal fibroblasts with potential of smooth muscle metaplasia. This process which is described by Leyendecker et al. as a second injury event, leads to the formation of adenomyosis. The initiation of the TIAR mechanism in endometrial stroma and the perpetuation of it, leads to the initial events in the development of both endometriosis and adenomyosis. (*30*)

1.3.6 Stem cell theory:

Another possible but not proven theory is stem cell seeding. The shedding endometrium also consists of endometrial stem cells. Because of Sampson's retrograde menstruation theory, some of the stem cells attach in the peritoneum. The theory is based on vaginal bleeding in 5% of neonate females, which is induced by transformation of the unborn' endometrium in late pregnancy. It transforms into a decidualized layer that desquamates after birth. Because of functional obstruction in new born babies it is assumed that this leads to regression of endometrial cells and their implantation in the peritoneal cavity. The implants survive until puberty , when estrogen production kicks in, they begin to proliferate. (*2, 31*)

1.3.7 Estrogen- and hormone dependency

Endometriosis is an estrogen dependent disease. Patients have elevated levels of estrogen and very low progesterone. The high level of estrogen leads to activation of estrogen-receptors and their specific gene regulation pathways. The main natural estrogen involved in endometriosis is Estradiol (E2), which is secreted by the ovary. Estrone (E1), estriol and estetrol play a minor important role. (*2*)

1.4 Female menstrual cycle:

The female menstrual cycle can be dived into two cycles, the ovarian cycle and the uterine cycle. The ovarian cycle only focuses on changes that happen in follicle production, whereas the uterine cycle describes changes in the endometrial lining. However, both cycles are divided into three different phases. The ovarian cycle is made up of the follicular phase, ovulation, and the luteal phase, whereas the uterine cycle consists of menstruation, proliferative phase, and secretory phase. (*32*)

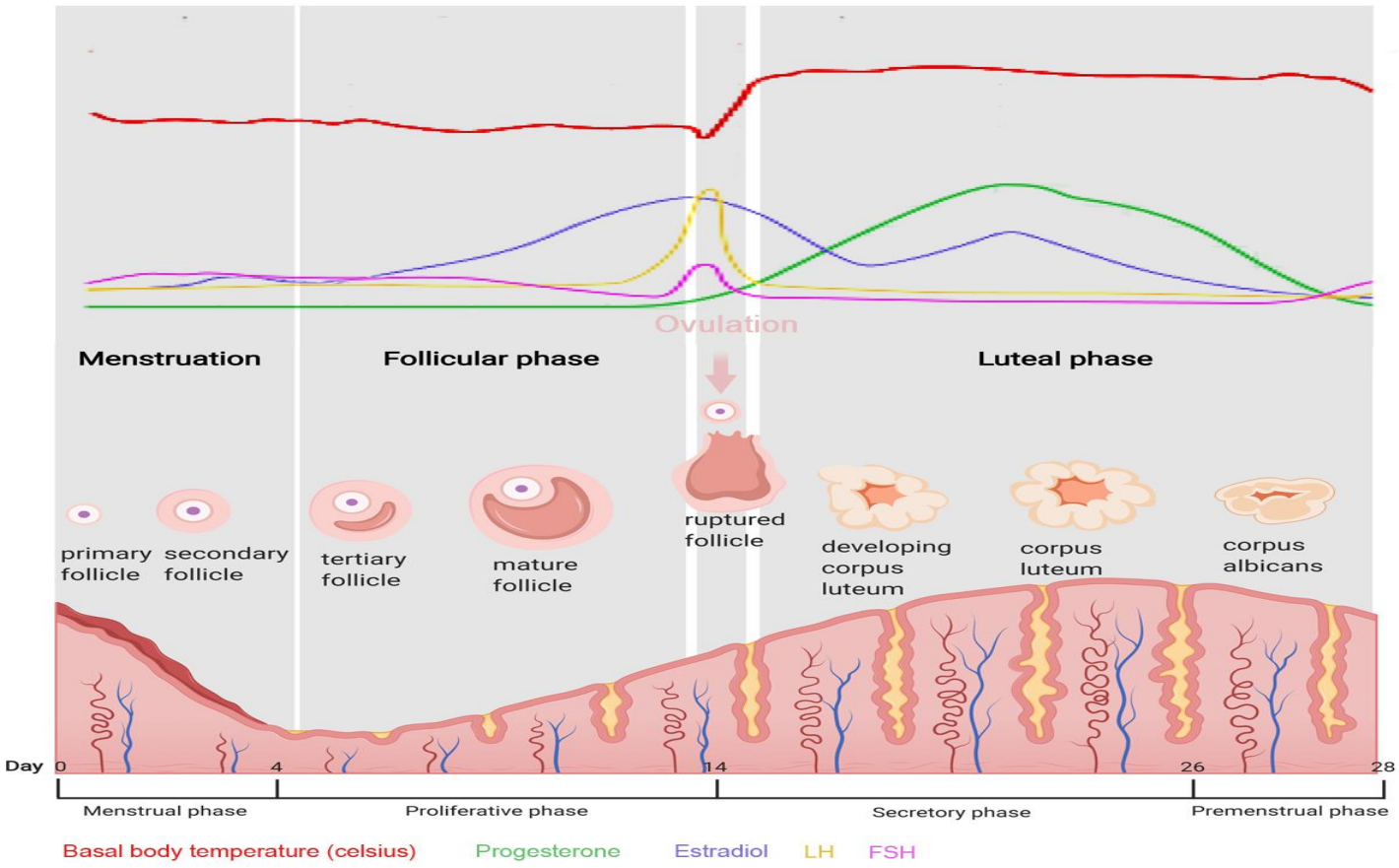
The follicular phase is strongly controlled by estradiol and ends with ovulation. In the follicular phase the follicles in the ovary mature. This process happens in different stage, initiation, recruitment, progression, pre-ovulatory maturation, and ovulation. In order for the follicles to mature FSH (Follicle-stimulating hormone) is secreted by the anterior pituitary. In the last days of the previous menstrual cycle FSH secretion begins. Through the rise of FSH levels five to seven tertiary-stage ovarian follicles are recruited to enter the menstrual cycle. These tertiary-stage follicles have been for a year during folliculogenesis. (*33, 34*)

The proliferation of granulosa cells in the follicles and the expression of luteinizing hormone receptors on these granulose cells, is stimulated by FSH induction. The influence of FSH, causes the activation of aromatase p450, therefore androgens are transformed into estrogen. The estrogen rising leads to an increased production of gonadotropin-releasing hormone. (GnRH). 8GnRH then leads to an increase in LH production LH rising ultimately leads to ovulation. As figure 3 shows, the second phase of the ovarian cycle is ovulation. A mature egg is now released from the ovarian follicles into the oviduct. During the follicular phase, estradiol suppresses release of luteinizing hormone (LH) from the anterior pituitary gland. After the egg has matured, it produces estrogen itself and the estradiol levels reach a threshold above which estrogen stimulates the production LH. This event is called the LH surge, as it is shown in figure 2, LH rise leads to ovulation.

The high estrogen levels, stimulate the growth of the endometrium and also the myometrium in the uterus. (*33*) Throughout the entire follicular phase, rising estrogen levels in the blood stimulates growth of the endometrium and myometrium of the uterus. (*35*)

After ovulation has occurred the luteal phase is initiated. If fertilization does not occur during ovulation, FSH and LH lead to transformation of the egg into the corpus luteum, which produces progesterone. The increased progesterone initiates the induction of estrogen

production again. Progesterone produced in the corpus luteum suppress FSH and LH production. Therefore, the level of FSH and LH go down fast, and the corpus luteum dies. When the corpus luteum has stopped producing progesterone, menstruation, the first phase of the uterine cycle, is triggered. (*36*) After menstruation has occurred, the second phase is initiated. The proliferative phase describes the growing of the lining of the uterus, due to estrogen. The maturing follicle produces estradiol E2, which leads to proliferation of endometrial cells, to form a new layer of the endometrium, which was shedded during menstruation. While luteal phase is occurring, the secretory phase is the last phase in uterine cycle. The progesterone produced by the corpus luteum leads to establishment of the proliferative endometrium in order to harbor an embryo and facilitate implantation. by increasing blood flow and uterine secretions and reducing the contractility of the smooth



muscle in the uterus it also has the side effect of raising the woman's basal body temperature. (*37, 38*)

Figure 2 menstrual cycle

1.5 Estrogen metabolism:

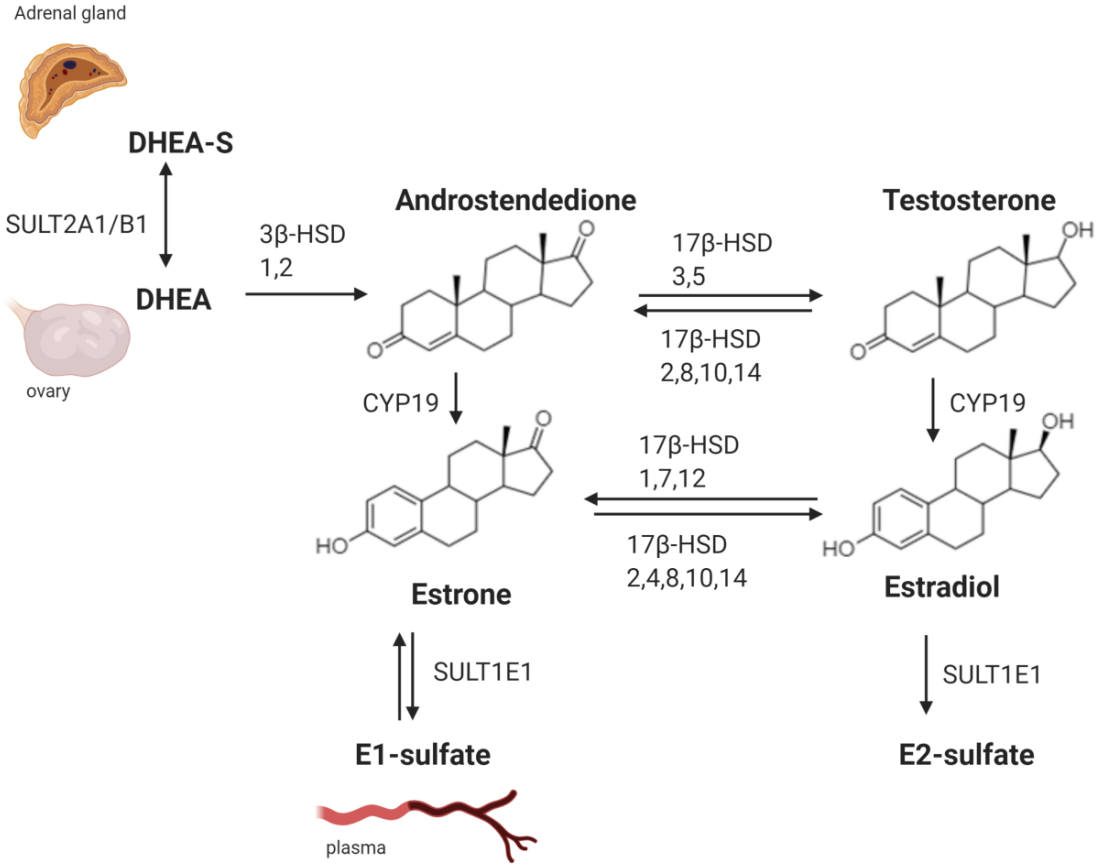


Figure 3 estrogen metabolism

Estrogen synthesis can happen through different pathways. However, their main production location is in the ovaries and peripheral tissue. Inactive precursors of adrenal dehydroepiandrosterone sulfate (DHEA-S), dehydroepiandrosterone (DHEA) and androstenedione can be used for local ovarian production. DHEA and estrone sulfate can be used to synthesize estradiol E2 via the aromatase pathway. DHEA-S, DHEA, androstenedione and testosterone can be transformed by steroid sulfatase 3-hydroxysteroid dehydrogenases (3-HSDs), aromatase and the reductive 17-hydroxysteroid dehydrogenases (17-HSDs). All these enzymes are also produced in the endometrium. Even so, the estrone sulfotransferase, which converts estrone sulfate to estrone. A small quantity of estrogens, mainly E2 is not only produced in the ovary but can also be locally produced by converting DHEA-S, DHEA, androstenedione and estrone sulfate. (*39*)

1.5.1 Estrogen production in endometriosis

Because of the increase of estrogen around ovulation, estradiol directly reaches endometriotic lesions in the peritoneum. The estrogen receptor pathways then lead to a proliferative tissue survival within the ectopic tissue. The lesions also produce aromatase and receptors for progestin and androgens, to sustain their estrogen production. When the secretory phase of the uterine cycle is reached progesterone P4 is mainly secreted by the corpus luteum, leading to a regulation of genes such as estrogen receptor alpha. Therefore, it acts as a growth inhibiting action through its receptors PR-A and PR-B. PR-B is found downregulated in ectopic tissue, while in uterine endometrium the transformation of estradiol to estrone is inhibited, by progesterone activity. In Endometriosis estradiol levels are high, due to progesterone resistance. The resistance might be because of the presence of PR-B inhibitor PB-A upregulation. (*2*) 17-HSD type 2 levels have also been found downregulated or with no expression at all in ectopic endometrium in ovarian and peritoneal endometriosis, and in deep-infiltrative endometriosis. 17-HSD type 2 usually, inactivates Estradiol E2. (*39*) Also, aromatase levels are found elevated in ectopic lesions, this correlates with dysmenorrhea and the high production of prostaglandin E2 and F2 alpha. Prostaglandin induces pain and excessive uterine contractions, which also causes dysmenorrhea. (*2*) Also, Cholesterol transport protein steroidogenic acute regulatory protein (StAR) is highly expressed in ectopic lesions, in contrary to eutopic endometrium. Especially, prostaglandin E2 stimulates the production of StAR via the binding of CCAAT/enhancer binding protein (C/EBP), beta binding to the cyclic AMP-responsive element-binding protein 1 (CREB) and cis-element. Cholesterol can then be used for estradiol synthesis. (*40*) First Cholesterol is converted into pregnenolone by the mitochondrial cholesterol side-chain cleavage enzyme (CYP11A1). It is not yet understood how the pathway really works, but there are two alternative enzymes for CYP11A1, CYP17A1 and HSD3B2. However, it has been shown, that ectopic endometrial cells produce P4 out of cholesterol. Even so P4 synthesis and expression of CYP11A1, CYP17A1 and HSD3B2, are enhanced by PGE-2, which means that, inflammation initiates de novo steroid synthesis in endometriosis. Furthermore, CYP19A1 enzyme catalyzes the transformation of A4 to E1 as well as testosterone to E2 Estradiol. Cytokines, TNFα and IL-6, stimulate CYP19A1 expression in endometriosis, which means that inflammation enhances endometriosis activity and survival. (*41*) Furthermore, because of CYP19A1 and HSD17B1 activities, the E2 level is enhanced. And the locally produced E2 induces cyclooxygenase 2 (COX-2) enzyme expression, therefore the synthesis of PGE-2 is increased. Furthermore, PGE-2 stimulates StAR and CYP1941 again, which means that E2 synthesis is further enhanced. (*40*) Figure 4 gives a quick overview on the mechanism.

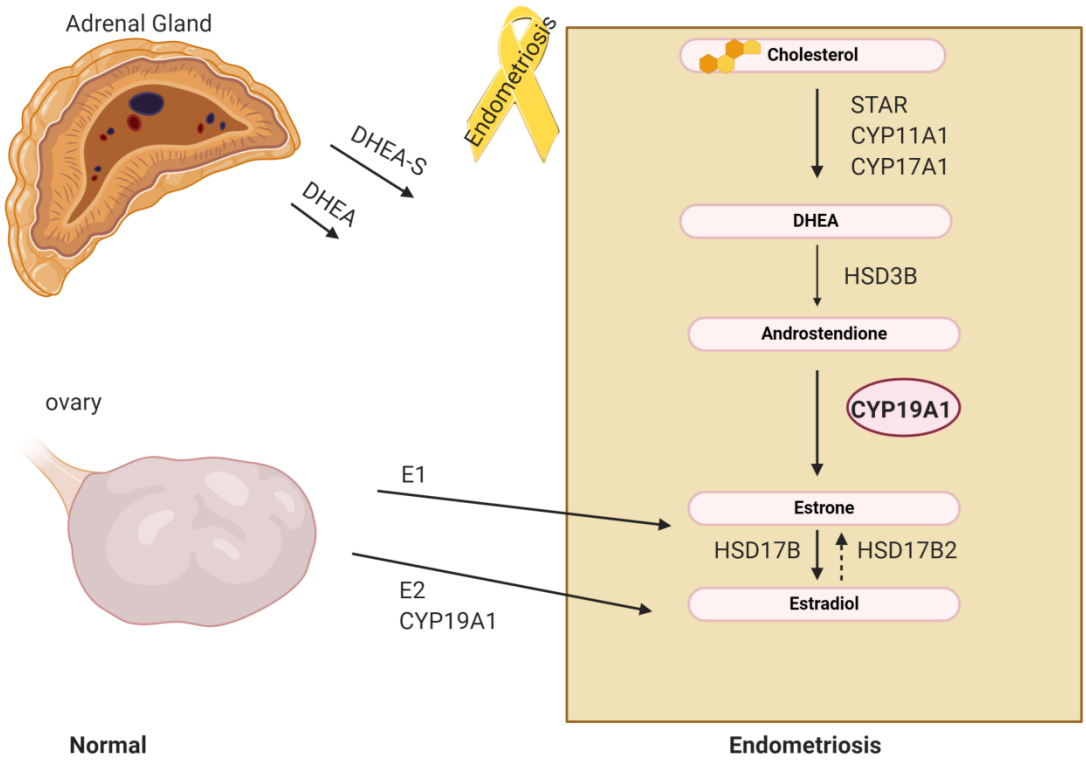


Figure 4 estrogen dependency pathway modified from(*40*)

1.6 Immune interactions in endometriosis:

Immunosuppression plays an important role in endometriosis development. CD4 T-cells secrete interleukins which may either stimulate or suppress immune activation. TH1 cells express primarily the pro-inflammatry cytokines IL-2, IL-12 and interferon γ. TH2 cells secrete rather anti-inflammatory cytokines IL-4, 5, 6, 10 and 13. In endometriosis the cell mediated immunity in the peritoneal fluid is suppressed by an increased production of IL-4 and IL-10. This leads to a decrease in T cell mediated cytotoxicity. In contrast to eutopic tissue, ectopic lesions produce cytokines to activate macrophages and suppress their phagocytosis, to evade immune reaction. This leads to a chronic inflammatory state, which further activates cycloocygenase-2 COX-2. The high COX-2 activity stimulates the activity of aromatase, which leads to high estrogen and proliferation. Endometriosis patients have elevated levels of cytokines, growth factors, T-cells, B-cells and macrophages in the peritoneal fluid. However, natural killer cell activity is decreased. The lesions form a constant blood supply through angiogenesis, for which they produce VEGF and TGF-beta and IGF (insulin growth factor) at high concentrations. In woman without endometriosis the immune system would eliminate lesions outside the uterine cavity, which leave the fallopian tube due to retrograde menstruation. In endometriosis patients the immune response must be altered or dysregulated. The missing immune surveillance could further promote endometriosis. (*42, 2*) An abnormal cell-mediated immunity (CMI) could lead to the growth and survival of ectopic tissue. Ectopic lesions produce ICAM-1, which is important in cell adhesion. The activity of natural killer cells could be disturbed by ICAM-1 expression, as well as expression of s-ICAM-

1. S-ICAM-1 binds to leukocyte-related ligands, which leads to decreased leukocyte-cell communication. TH2 cells also stimulate CMI, by the release of IL-4 and IL-10. The inflammatory response leads to angiogenesis, adhesion, neuronal infiltration and high oxidative stress. In order for the ectopic cells to establish these conditions, the immune response must be down regulated in the peritoneum. T Lymphocytes and also NK cells are poorly recruited. (*2*) But Mier-Cabrera et al. proved that the weak cytotoxic response is not because of low concentration of T-cells, but because of a different T-cell function. (*43*) NFκB transcription factor also enhances the pathogenesis by increasing inflammation and invasion, which further leads to angiogenesis and down-regulating apoptosis. Accumulation of iron may also promote the chronic activation of NFκB. Peritoneal macrophages secrete IL-1, which leads to an overproduction of IL-8, TNFα and interferon y. These cytokines further lead to stimulation of macrophages, which also produce prostaglandin E2 and VEGF. (*2, 41, 44*) The high concentration of activated macrophages and lymphocytes in the peritoneal fluid may lead to high levels of TNFα. Also, the eutopic endometrium has enhanced proliferation and has a higher chance to grow and survive outside the uterus than endometrium from non-Endometriosis patients. Also the number of lymphocytes in eutopic and ectopic endometrium is also enhanced. (*45*) Also, Antsiferova et al. showed that, the levels of IL-4 and IL-1 in peripheral blood lymphocytes was significantly higher, than in the control group of healthy women. Il-4 had the highest expression in ectopic endometrial Lymphocytes. Il-4 synthesis is also enhanced in intracellular levels. Because, of the high increase of IL-4 TH2 cells are activated. The increase of TH2 cells leads to an activation of local humoral immune responses and autoantibody production. (*45*)

1.6.1 TNF-α:

TNF-α or Tumor necrosis factor is a cell signaling cytokine, which is a initiator in systemic inflammation and activates acute phase reaction. It is produced by activated M2-macrophages, but also by CD4+ lymphocytes, NK cells, neutrophils, mast cells, and eosinophils. (*46*) TNF-α is the main regulator of immune cells, it leads to induction of fever, apoptosis, cachexia , inflammation and also response via IL-1 and IL6. (*47*)

TNF has two receptors, TNFR1 and TNFR2. TNFR1 is expressed in most tissues, while TNFR2 is normally produced in immune cells. (*48*) The TNFR1 pathway has various activation pathways: One is the NFκB signaling, which also plays a role in endometriosis. By binding of TNFα to its receptor the protein kinase IKK is activated. The NFκB inhibitor IkBα is phosphorylated by IKK and degraded. NFκB is now free to translocate into the nucleus and initiate transcription of genes involved in cell survival, growth and antiapoptotic factors. Another pathway is the activation of the MAPK signaling cascade. By activating of the ERKs and JNK, JNK and c-JUN dimerize and activate genexpression of proteins involved in cell differentiation and proliferation. (*49, 50*) MAPK signaling is associated with endometriosis, by the enhanced sensitivity to pain, patients experience. Neurons and glia normally activate MAPK pathways in response to inflammatory pain and neuropathic pain. Woman with endometriosis often

experience neuropathic pain levels. P38 MAPK plays a role in the mechanism of chronic pain development. (*51*)

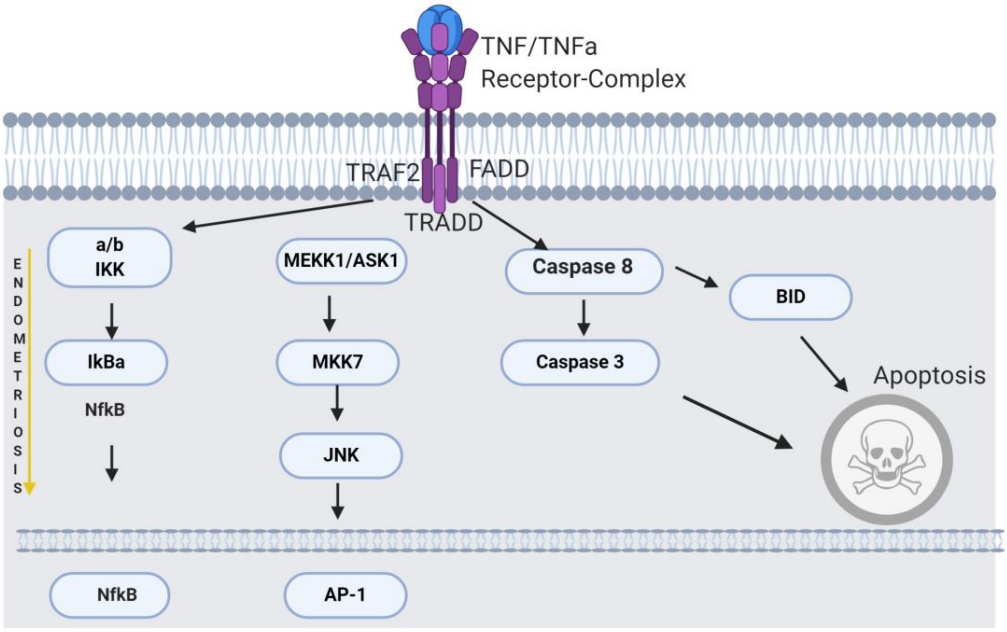


Figure 5 signaling pathway of TNFR 1

TNFR1 is also involved in apoptosis signaling, but signaling is weak in comparison with its inflammatory pathways. Also NFκB activation inhibits TNF-α stimulated pro apoptotic effects. (*50*) Cytokines and ROS (reactive oxygen species) can lead to enhanced NFκB signaling in endometriosis, which inhibits apoptosis. (*2, 52*) Endometrial cells usually express NFκB and activate it, upon stimulation by TNF-α. NFκB is mostly activated during menstruation period in glandular epithelium and epithelium of the endometrium. TNF-α stimulates the activation of endometriotic stromal cells, which leads to high level of IL-8 and also RANTES, which plays an important role in chemotaxis of endometriotic cells and therefore in the pathogenesis of endometriosis. (*53*) Also, González-Ramos et. Al showed that NFκB signaling is constantly activated in endometriosis lesions. (*53*)

TNF-α has a menstrual cycle-dependent expression pattern, which is important for cell differentiation and tissue remodeling.

Furthermore, stromal endometrial cells produce Prostaglandin F2α upon stimulation of TNF-α. Prostaglandin is usually secreted from the uterus in luteal phase, when the corpus luteum is degraded. TNF-α stimulates the production of Prostaglandin F2α in stromal cells, but not in epithelial cells of the endometrium, which means that the induction leads to initiation of ovarian oxytocin and uterine PGF2α to perform luteolysis of the epithelial cells. The production is initiated by activation of phospholipase A2 and arachidonic acid release. Also, TNF-α directly regulates prostaglandin endoperoxide H synthase (cyclooxygenase) COX, which converts arachidonic acid to prostaglandin F2α. While COX-1, one isoform of COX, is produced in endometrial cells without further stimulation, COX-2 is only expressed upon oxytocin and

cytokine stimulation. TNF-α is one of the main cytokines to initiate COX-2 activity. (*54*) Furthermore TNF-α levels in the serum of endometriosis patients is significantly higher, than in healthy women. (*55*) The expression rate of COX-2 in ectopic endometriotic cells is higher than in eutopic endometrium of endometriosis patients. COX-2 and VEGF play an important role in endometriosis progression. Ectopic lesions must successfully perform angiogenesis to have blood supply for their growth. VEGF was found upregulated in the peritoneal fluid of endometriosis patients, as well as COX-2 levels. Neovascularization is needed to proliferate and invade into ectopic sites, which is induced by whether VEGF and COX-2. Peritoneal macrophages produce COX-2 and provide constant stimulation for ectopic lesions. It has been found, that COX-1 is higher expressed in eutopic endometrium, while COX-2 is highly produced in ectopic cells. Stimulation with TNF-α in invitro studies showed, that COX-2 production in ectopic and eutopic endometrium was enhanced. Stimulation with Il-1β on the other hand, increased COX-2 expression only in ectopic cells. The production of COX-2 was stimulated by the p38 MAPK pathway, either by Il-1β or TNF-α. (*56*)

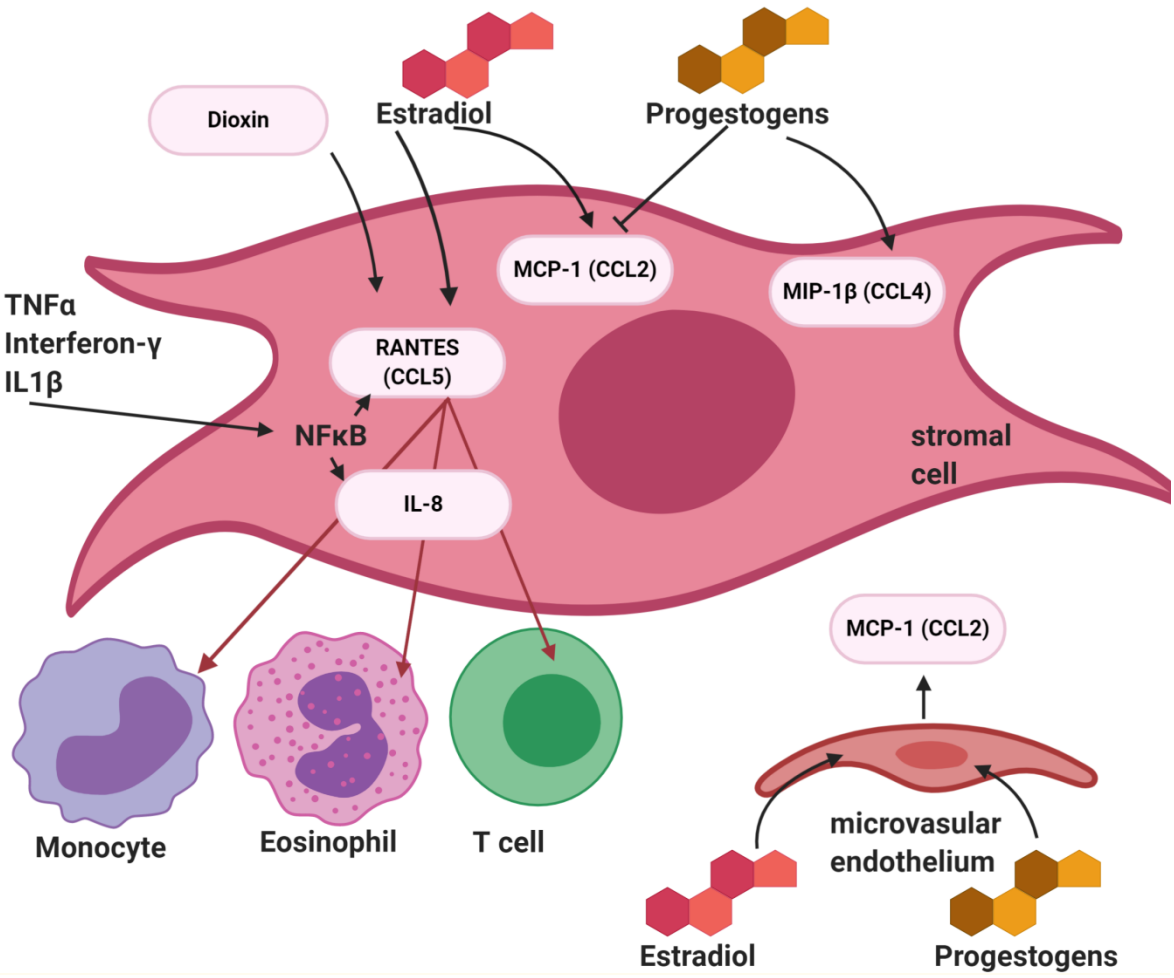


Figure 6 endocrine and paracrine regulation of endometriosis modified from (57)

Figure 6 shows, that leukocytes are attracted by released chemokines from endometriotic cells in response to estradiol stimulation, but also in response to TNF-α. Endometriotic cells

stimulate via steroids MCP-1, which is a representative for CC molecules, as well as RANTES is. Estradiol and Dioxin induce the expression of MIP, which plays an important role in endometriotic cell invasiveness. Therefore patients with endometriosis have an aberrant CC chemokine response to hormones in stroma and also in microvascular endothelium, which could explain the therapeutic failure of pain management with progesterone or hormonal contraceptive treatment. (*57*) TNF-α and estradiol together induce Il-8 production by NFκB signaling. Synthetic progesterone levonorgestrel even leads to an increase in chemokine , such as Il-8, release, which counteracts the therapeutic effect. (*57*)

Further, apoptosis is inhibited by numerous pathways stimulated by chemokines or estrogen in women with endometriosis. As figure 8 shows, estradiol stimulation leads to BCl2 production, which is an antiapoptotic molecule inhibiting apoptosis. The enhanced activation of ERK and AKT signaling by estradiol and NFκB induced inhibition of apoptosis upon stimulation by TNF-α, also leads to decreased cell death. TNFα further activates matrix metalloproteases, which further down on their pathway inhibit Caspase-8 a crucial initiator for mediated cell death. (*57*)

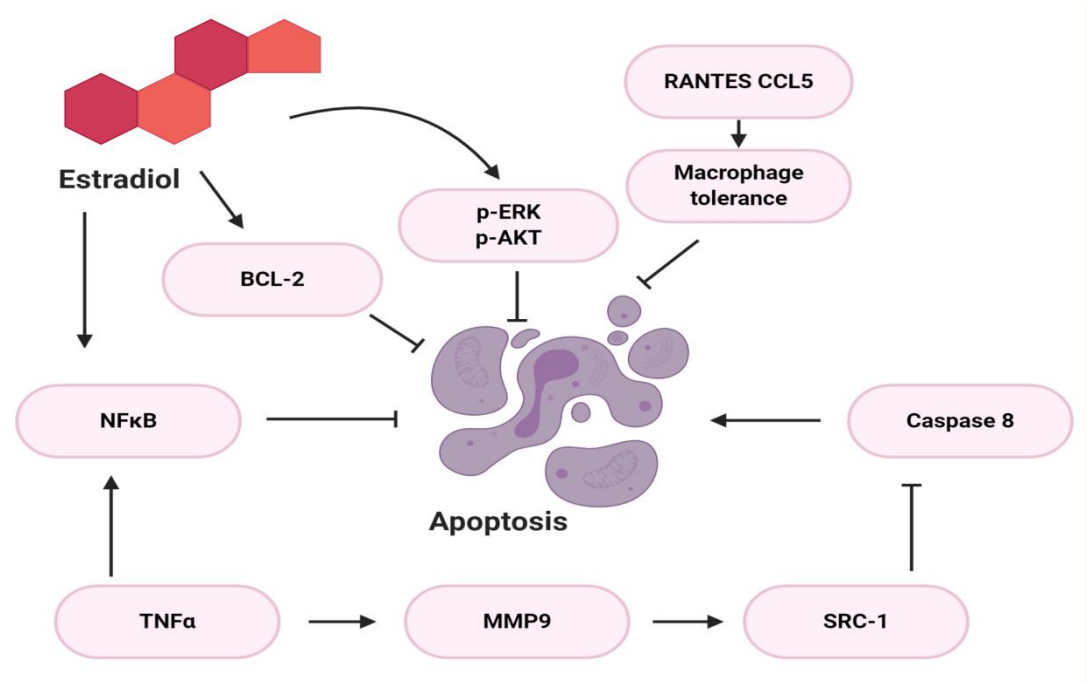


Figure 7 apoptosis inhibition in endometriosis modified from (57)

1.6.2 Interleukin 4-

The development of endometriosis is associated with the high cytokine production and activation of phagocyte cell activity. In ectopic endometrial cells intracellular level of Il-4 synthesis is significantly higher than in controls. Also, autoantibodies including anti endometrial and antispermal antibodies are enhanced in the serum of women with endometriosis. The augmentation of IL-4 expression leads to activation of TH2 lymphocytes, which produce these antibodies, which can lead to infertility. Lymphocytes infiltrating endometriotic lesions have elevated Il-4 levels. Il-4 is the one of the main cytokines to play a

role in differentiation and maturation of B lymphocytes, which are also highly activated in ectopic endometrial tissue. The augmentation of B cells could be a possible link to an autoimmune pathology. The growth of peritoneal ectopic endometrial tissue is accompanied with humoral immune response activation due to the high amount of Th2 lymphocytes activated by a high intracellular synthesis of IL-4. (*45*) Interleukin 4 binds on non-immune cells to its receptor, which can activate various signaling cascades. OuYang, Hirota et. al showed that IL-4 stimulates the phosphorylation of p38 MAPK, SAPK/JNK, and p42/44 MAPK, which leads to proliferation of endometriotic cells. (*58*) Also if cells are treated both with Interleukin 4 and TNFα they show a synergistic effect on proliferation. Enhanced activity of macrophages in the peritoneal fluid of endometriosis patients produce pro-inflammatory cytokines and further sustain themselves by perpetuating autocrine inflammation. (*58*) Anti-inflammatory cytokines like Interleukin-4 might have insufficient control over inflammation signaling and their altered expression could eventually cause over-compensation potentially resulting in the increase of pro-inflammatory cytokines. Figure 9 shows that anti-inflammatory cytokines lead to development of endometriosis. In the endometriotic microenvironment, endometrial cells, peritoneal mesothelial cells and immune cells (such as macrophages, T-cellsand natural killer cells) produce anti-inflammatory cytokines like Interleukin-4. These cytokines are initiators of EMT (epithelial to mesenchymal transition), which is needed for invasion and metastasis. Also, anti-inflammatory cytokines induce mesothelial-to-mesenchymal transition (MMT), fibroblast-to-myofibroblast transdifferentiating (FMT), smooth muscle metaplasia (SMM), fibrogenesis and angiogenesis, and impairment of immune surveillance of the ectopic tissue. In EMT cells lose their polarization of the cytoskeleton and loosen their cell-cell contact, which is actually the first step. Genexpression of epithelial cells is stopped and mesenchymal gene expression is initiated. TGF-β is one of the main inducers of EMT. As mesenchymal cells , endometriotic cells can migrate and invade other tissues. (*59*)

Protein levels of OCT4 are dose-dependently increased by TGF-β1 stimulation, further the genexpression of SNAIL and N-cadherin as well as silencing of endogenous OCT4 are enhanced. OCT4 leads to a suppression of TGF-β1-induced effects. TGF-β and OCT4 together initiate the EMT process in endometriosis. Endometriotic mesenchymal cells can then undergo fibrogenesis via the WNT/β-catenin signaling pathway, via a paracrine signaling of WNT and TGF-β. Activation of platelets, leads to promotion of EMT and FMT in endometriosis due to the secretion of TGF-β1. This may result in enhanced cell contractility, collagen production and fibrosis. Furthermore, if endometriotic cells get in contact with activated platelets, expression of alpha smooth muscle actin (α-SMA) and markers of differentiated smooth muscle cells is enhanced (*60*). Smooth muscle metaplasia primarily happens in peritoneal, ovarian, extragenital, and pleuropulmonary endometriosis (*61*). The protein tyrosine-kinase FAK plays an important role in EMT progression in endometriosis within the myometrium, also called, adenomyosis. As a component of Integrin complexes FAK regulates the cytoskeleton and cell motility, and participates in numerous cells signaling. The overexpression of FAK leads to involvement and initiation of PI3K/AKT signaling, transforming growth factor β (TGF-β), hepatocyte growth factor and MAPK/ ERK pathways, which are involved in the EMT process.

FAK also regulates the expression of epithelial-associated markers (E-cadherin and cytokeratin) and mesenchymal-associated markers (N-cadherin and vimentin). In EMT epithelial markers especially E-cadherin is downregulated and the production of mesenchymal markers like N-cadherin is increased. Without E-cadherin the cell-cell contacts are loosen up, while N-cadherin promotes cell motility. Furthermore, FAK regulates expression of matrix metalloproteinases (MMPs), which degrade proteins of the extracellular matrix to facilitate migration. FAK also shows anti-apoptotic features by inhibiting p53 anti-apoptotic properties. (*62*)

1.7 The immunologic environment in endometriosis:

Immune cells infiltrate endometriotic lesions, because of the chemokines the ectopic tissue secrets. Both endometriotic cells and immune cells produce prostaglandins and pro inflammatory cytokines and inhibit the expression of anti-inflammatory ones. Because of the huge number of erythrocytes entering the peritoneal cavity due to retrograde menstruation, iron accumulation in peritoneal macrophages is a common situation in endometriosis patients. This leads further to oxidative stress in endometriotic lesions and the peritoneal fluid. Due to this stabilization of the lesions estrogen synthesis begins, because of aromatase p450 pathway and downregulation of 17-β hydroxysteroid dehydrogenase type II. Once neoangiogenesis and infiltration in nerve fibers was successful the endometriotic lesion has created its own altered microenvironment to further grow and spread via the lymph and blood system.

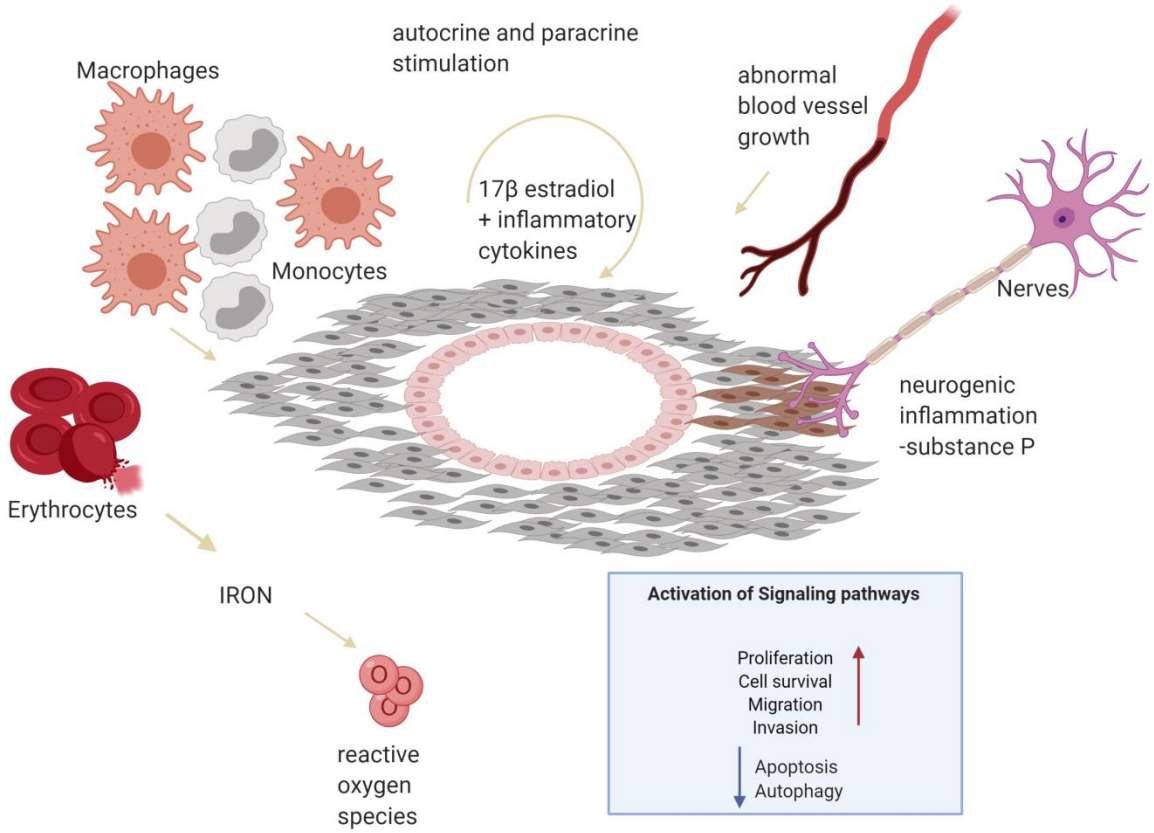


Figure 8 microenvironment of endometriotic lesions modified from (63)

Figure 8 gives an overview on the endometriotic environment. This altered environment is special and unique in endometriosis, created to sustain proliferation and estrogen synthesis of the lesions. This special environment leads to activation of kinase signaling pathways, that facilitate growth. (*63*) Heme and iron lead a pro-oxidant role in endometriosis development. The oxidative stress created leads to DNA damage and a decreased level of DNA repair , which leads to progression of the disease. (*64*) The high level of ROS also facilitates adhesion. (*65*) The activated macrophages in endometriotic lesions produce TNF-α , IL-1 and IL-6 which stimulates the activation of genexpression of IL-8 and RANTES, which further activates more macrophages and also T-lymphocytes and eosinophils. TNF-α and IL-6 together promote the expression of aromatase p450 and 17β-hydroxysteroid dehydrogenase. (*66*) Macrophages elevate ROS levels further, resulting in a hyper oxidative environment. (*65*) Because of the high NO-level produced by activated macrophages, which migrate to the lumen of the oviduct, the embryo is attacked and implantation is hindered. The high iNOS expression is one of the main reasons for infertility. (*67*) The iron overload leads to increased proliferation of the ectopic cells due to activation of genexpression of proteins involved in iron metabolism and oxidative stress. Some of these genes are even endometriosis specific, which encode for proteases, adhesion molecules, signaling molecules, transcription factors, hormones, cytokines, pro-inflammatory factors, growth-factors and molecules involved in the cell cycle, stress and detoxification. (*68*) Iron is essential for proliferation, because of catalysis of oxygen sensing, energy metabolism and cell respiration. (*2*) A cell without iron can't transition from G1 to S phase of the cell cycle. (*69*) Iron and ROS together activate OS response genes like hemeoxygenase and detoxification enzymes, which are already affected in endometriosis, due to NFκB upregulation and stimulation of COX-2 expression. Iron also regulates production of genes produced in macrophages, for example proteins protecting lipids from peroxidation. (*70*) The Iron overload in endometriotic lesions leads to progesterone resistance due to down regulation of hormone receptors, progesterone responsive genes and progesterone-regulated genes. (*71*) In the peritoneal fluid of endometriosis patients a high amount of iron-laden macrophages can be found. (*72*) Macrophages release ferritin, which lead to higher iron concentrations. (*73*) High iron concentrations are toxic because of Fenton reaction, which leads to deregulation of cellular processes, cell dysfunction and cell death. Fenton reaction creates free radicals like OH and ONOO- as a product of the reaction between NO and O2-. (*74*) The Fenton reaction leads to an increase of OH radicals, which upregulate NFκB, which than inhibits apoptosis and induces genexpression of growth proteins and angiogenic factors, as well as COX-2 production and increase of adhesion molecules. (*2*)

1.7.1 Neutrophils in endometriosis:

Cytokines like IL-8 and CXCl10 as well as VEGF which are produced by peritoneal neutrophils, promote endometriosis progression. IL-17A, which is also expressed in ovarian endometrioma, increases secretion of CXCL1, which recruits more neutrophils, inducing a chronic inflammatory condition, which is a main symptom in endometriosis.(13)Also neutrophils have been found in a significant high amount in peripheral blood in endometriosis patients. (*75*)

1.7.2 Macrophages:

Macrophages are not only increased in ectopic lesions but also in eutopic endometrium of endometriosis patients. They are activated by NFκB and produce TNF-α in a high amount, as well as IL-6, IL-1β and IL-4. Due to their secretion of VEGF, endometriotic lesions can undergo angiogenesis. Tie2 (tumor associated) macrophages have an enhanced pro-angiogenic activity. These macrophages are attracted by the endometriotic lesions. GM-CSF and the action of Stat3 initiates the growth of the lesions. Endometriotic macrophages have stopped to produce their cluster of differentiation molecule CD36, which leads to decreased phagocytotic activity. Annexin A2 on macrophages, which is normally used for phagocytosis is reduced in endometriotic macrophages. Especially M2-macrophages (activated by TH2 cells) play an important role in endometriosis. (*75*) M1-macrophages are significantly decreased in peritoneal fluid of patients, whereas M2-macrophages are enhanced. A mouse model even showed, that injection with M2 macrophages promoted endometriosis lesions growth. (*76*) M2 macrophages promote the production of MMP-9 and MMP-27 Matrix-metallo-proteases, which leads to increased matrix remodeling. Endometriotic macrophages infiltrate nerve fibers, mediated by estradiol crosstalk. (*75*) They express estradiol receptors on their surface and migrate towards nerve fibers in an estrogen dependent way. Nerve fibers usually secrete E2 for neuroprotective manners. Because endometriosis assisted macrophages recognize ectopic lesions as wounds, they infiltrate nerves as they would do in nerve injury conditions. Nerve fibers in endometriotic lesions are not injured, but create a similar environment as traumata would do. Estrogens produced by endometriotic cells act on nerve fibers within lesions, to induce expression of CSF1 and CCL-2, recruiting macrophages. Furthermore Estradiol acts on the attracted macrophages to increase production of brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3, which leads to enhanced neurogenesis. (*77*) This process is shown in figure 9:

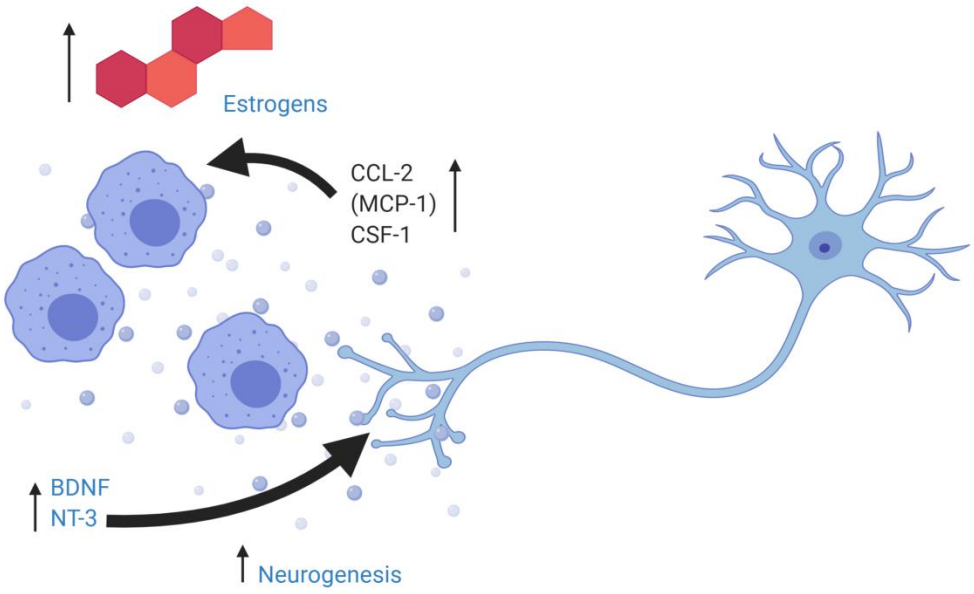


Figure 9 estrogens stimulating macrophage infiltration into nerve fibers modified from (77)

Estrogen receptor Eα and IL-6 crosstalk, leads to recruitment of more monocytes, which will then maturate and differentiate into macrophages with estrogen receptors. These macrophages produce IL-6 again, which promotes a positive feedback loop. Binding of estrogen on ER-α regulates the IL6 promotor via the activation of NFκB and CEBPβ. Both ERα and ERβ receptors are expressed on the surface of endometriotic macrophages. Stimulation of 17-β estradiol leads to expression of TNF-α and IL-6. A peripheral axon injury leads to secretion of neuron, derived exosomal miR-21-5p, which is further phagocytosed by macrophages, which mediate the infiltration of macrophages into peripheral nerves. Estrogen signaling is proven to be the cause of the exosomal secretion of miR-21-5p by neurons. (*78*) Estrogen also stimulates the release of colony-stimulating factor 1 (CSF1) and C-C motif ligand 2 (CCL2) from peripheral nerves, which increases infiltration of macrophages into the endometriotic lesion. M2 macrophages are polarized by uptake of TH2 secreted IL-4 via their IL-4 receptor. Furthermore, regulation of CCL2 in the peritoneal cavity is estrogen dependent and leads to polarization of M1 macrophages towards M2 macrophages due to the CCL2/CCR pathway. Estrogen also acts on nerve fibers to secrete LIF, IL1 and PAP3, which promote the recruitment of macrophages. Estrogen increases the release of CSF1 and CCL2 to enhance infiltration of macrophages towards peripheral nerves. Interaction of CCL2 activates the polarization of M2 macrophages. (*79*) Estradiol also can lead to secretion of NGF by macrophages, which promotes aberrant neurogenesis and therefore pain sensation due to neural hypertrophy and increased neural density in the endometriotic lesion. Due to this neurogenesis there is an imbalanced distribution of sensory, sympathetic and parasympathetic nerve fibers. NT3, NGF and BDNF are secreted by macrophages upon estradiol stimulation. Furthermore, estradiol secretion in endometriotic lesions leads to expression of NT3 receptor, tyrosine kinase receptor B (TrkB) and the receptor of BNDF on sympathetic neurons, which then promotes neuronal survival and sympathetic axonal growth in the endometriotic lesions. Further Semaphorins 3F and 3C are overexpressed in the endometriotic lesions, either by macrophages or due to the high estrogen concentration. Semaphorines normally regulate the axon migration, axonal growth and guidance, leading to an aberrant innervation in endometriosis. (*79*) VEGF secretion also plays an important role in axonal outgrowth. VEGF acts as a neurotrophic factor, by stimulation via its receptor VEGF/flk1. Flk1 is up-regulated in peripheral ganglia, whenever axonal injury happens. Flk1 is known to be involved in the pathogenesis of endometriosis. VEGF is also transported retrogradely into the peritoneal cavity. As it is known activated macrophages produce VEGF in endometriosis, by the regulation of estrogen. Macrophage-derived prostaglandin E2 increases the level of estrogen in endometriosis, which further is initiating the secretion of VEGF by macrophages. (*79*)

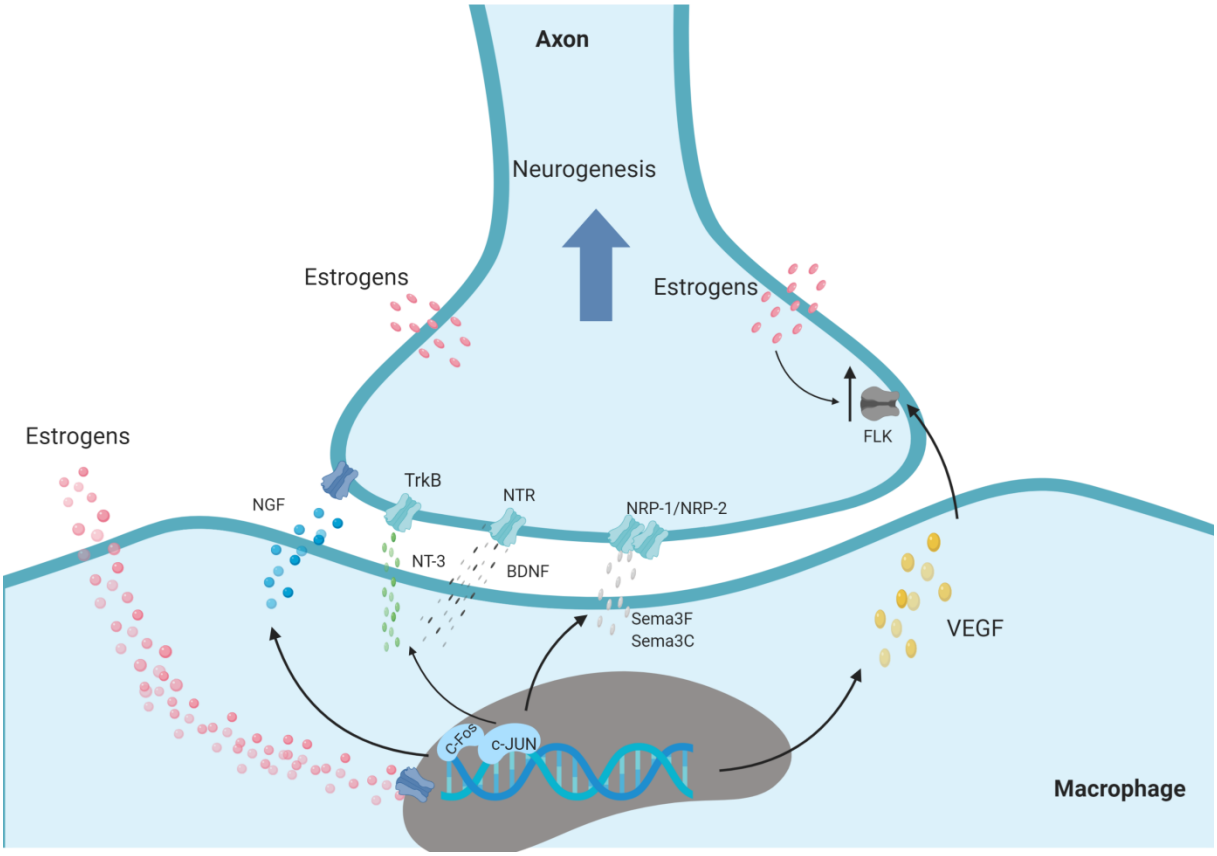


Figure 10 neurogenesis in endometriosis modified from (79)

Figure 10 shows how macrophages, induced by estrogen, upregulate NGF secretion. Expression of NT3 and BDNF is also enhanced due to estrogen. The semaphorines 3F and 3C secreted by macrophages reduce the sympathetic innervation. Lastly VEGF leads to dysregulation of nerve growth factors. (*79*)

1.7.2.1 Macrophage plasticity

Macrophage plasticity could be an important role in endometriosis development. CCl2 and CSF-1 secreted by nerve fibers along endometriotic lesions attract macrophages and strengthen macrophage migration. Even so the attraction could lead to the polarization from M1 status (phagocytotic pro-inflammation) towards the M2 (anti-inflammatory) state. (*80*) Therefore this could mean that endometriotic lesions attract nerve fibers themselves, so that the nerves can help in the recruitment of more macrophages. The different pathways of recruitment are shown in figure 11. A possible mechanism is shown in a. The secretion of MCP-1 and RANTES in the endometriotic lesion attracts macrophages, whereas in b CCL-2 and CSF-1 , which are secreted from nerve fibers in the endometriotic lesions recruit the macrophages infiltrating the lesion, which also changes their polarity. (*80*) The third alternative is mediated by Sema3A and NRP-1, which also play a role in the estradiol crosstalk between nervous cells and endometriotic macrophages. (*79*) Semaphorine 3A is a guidance factor for neurons and functions in axonal elongation restriction and growth cone collapse through its binding towards neuropilin-1 (NRP-1) and PlexinA1. (*81*) Since Sema3A has been detected in peritoneal and deep infiltrating endometriosis it is clear that there is a cross-mediating activity

in the aberrant innervations by it. Casazza et al. showed that NRP-1 is downregulated on the surface of of macrophages in a hypoxic environment, whereas the NRP-1 expression was significantly higher in a normoxic environment. (*82*) Since endometriotic lesions create a hypoxic environment, endometriotic macrophages could change their polarity upon SEMA3A/NRP signaling. Rivera et al. even proved that Sema3A/NRP-1 signaling is involved in macrophage reprogramming. (*83*)

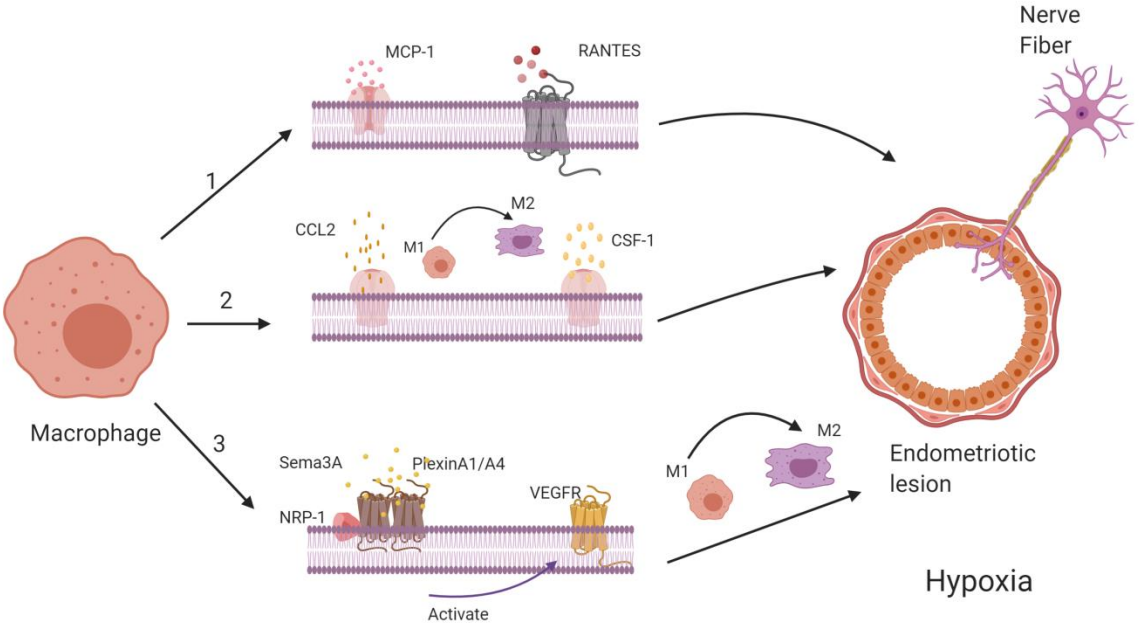


Figure 11 macrophage differentiation in endometriosis modified from (80)

Macrophage nerve signaling is also mediated by neurotrophins. Some of them like neurotrophin-3 (NT-3) are found overexpressed in ovarian endometriosis and deep infiltrating endometriosis. Even so peritoneal fluid of endometriosis patients contains high levels of BDNF and NT-3 suggesting an estradiol guided innervation, as mentioned above. This innervation leads to focal inflammation due to over secretion of pro-inflammatory cytokines by the macrophages. The focal inflammation further is stimulating the peripheral nerve endings, which start secretion of pro-inflammatory neuromodulators leading to peripheral neuroinflammation. Secretion of TNF-α by macrophages induces the sensory nerves to a constant induction of the action potential via the TRPV1 (transient receptor potential vanilloid 1) This is mainly through overexpression in patients of voltage-gated sodium channels. These channels can be modulated by TNFα. (*80*) Opioids can act as anti-inflammatory agents by inhibiting the channels, which was shown by Tang et al. (*84*) Norepinephrine from sympathetic nerve fibers are binding the Estradiol β2 receptor on macrophages, which leads to activation of PKA signaling and thus stimulating TNFα mediated inflammation in endometriotic lesions. In sensory nerve fibers of peritoneal and DIE the neuropeptide SP was found upregulated. (*80*) SP stimulates the release of TNFα, Il-6 and Il-8 by macrophages. (*85*) Therefore inflammation is starting all over again, leading to a vicious cycle.

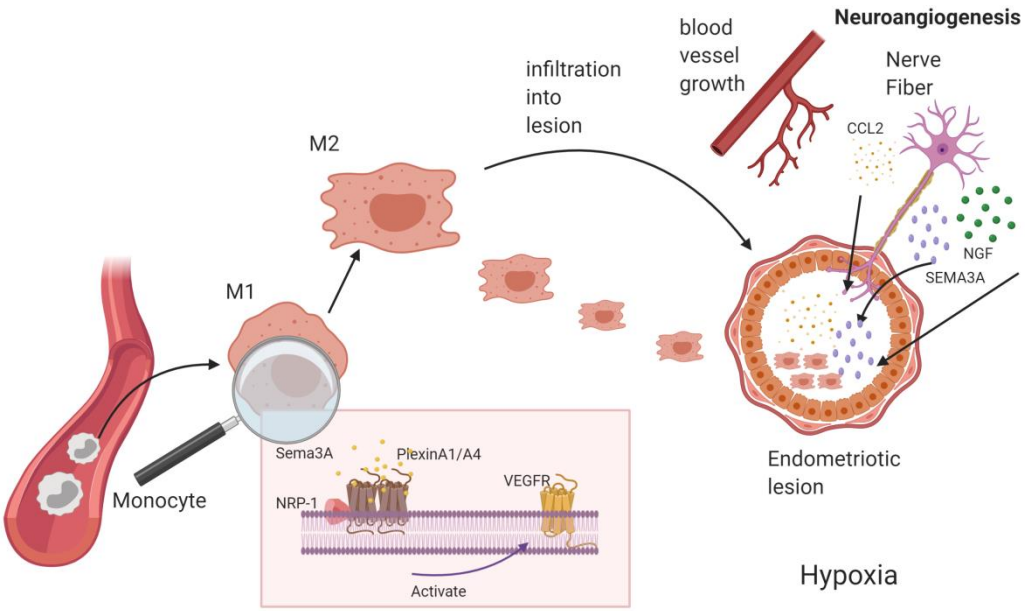


Figure 12 hypoxia leading to M2 polarization modified from (80)

Wu et al. propose a possible mechanism for the establishment of neuroinflammation in endometriosis. Although retrograde menstruation theory still remains as a theory it could be that endometriotic tissue transported into the peritoneum is already inflamed, thus creating an inflammatory microenvironment. Which further leads to macrophage infiltration and hyperinnervation. First M1 macrophages are migrating towards the endometriotic lesions to phagocytose and destroy them. Somehow phagocytosis is impaired and the macrophages assist the progression of the disease. This might be due to secretion of chemoattractant from the nerve fibers that mediate the transformation of M1 macrophages towards M2 macrophages. Therefore macrophages in the lesions secrete peptides promoting axonal growth and innervation. (*80*) It is not known what causes impairment of phagocytosis, but prostaglandin E2 impairs the phagocytic ability of peritoneal endometriotic macrophages via suppression of annexin A2. (*86*) The impairment of phagocytosis leads to further inflammation, because debris is not degraded. Qi Xie et al. showed that SIRP-α in peritoneal macrophages from endometriosis patients is significantly higher. (*87*) SIRP-α mediates negative phagocytosis in macrophages and binds CD47 as a receptor. (*88*) By binding to CD47 a so called "don't eat me signal" is created, thus leading to impairment of phagocytosis. CD36 on the other hand acts a positive regulator in phagocytosis promoting degradation of endometrial tissue. In macrophages isolated from peritoneal fluid from patients CD36 expression is significantly downregulated, thus leading to a non-functioning phagocytosis. It is not known, what causes this aberrant expression of SIRPα and CD36, but cell culture experiments showed that treatment of macrophages cell line THP1 with endometriotic tissue homogenate increased SIRPα and inhibited CD36 expression. (*87*) Prostaglandin E2 inhibits the expression of CD36 through its EP2 receptor dependent signaling pathway. (*89*) Moreover elevated heme concentrations lead to the impairment of phagocytosis. (*90*) Lysis of erythrocytes in the peritoneum leads to a higher concentration of heme in the peritoneal fluid of patients compared to the control. (*90*) Moreover also autophagy is altered in an impairing

way in endometriotic cells. The alteration in mTOR dependent downregulation of autophagy also leads to an inhibition of autophagy-dependent degradation of proteins and programmed cell death. Endometriotic cell use autophagy inhibition to escape from immune surveillance and implant themselves easily in ectopic sites. High levels of IL-15 are secreted in endometriotic cells to induce proliferation and inhibition of apoptosis in an autocrine way, but Il-15 also directly inhibits NK-cells killing activity through a paracrine way. By decreasing endometriotic autophagy, reactivity to IL-15 signaling is enhanced thus further leading to impairment of NK-cells activity. High levels of 17β estradiol mediate mTOR dependent inhibition of autophagy. One major force promoting autophagy-inhibition is hypoxia. Since endometriotic tissue create a hypoxic environment, through cyclic bleeding and therefore lysis of erythrocytes releasing high levels of heme, which is further oxygen dependently degraded into biliverdin, carbon monoxide and iron, oxidative stress is one driving force behind autophagy inhibition. (*91*)

1.8 Hypoxia in endometriosis:

When retrograde menstruation occurs, endometrial cell faces a hypoxic environment due to loss of blood supply. Hypoxia-inducible factor (HIF) consist of 2 subunits. (*92*) HIF-α subunit which is only accumulated during hypoxia is found upregulated in endometriotic stromal cells. HIF-α promotes steroidogenesis and enhanced estrogen responsiveness. (*93*) It is known that endometriotic cells are able to synthesize estrogen through cholesterol accumulation and aberrant expression of Star enzyme. Star is upregulated by prostaglandin E2 (PGE2) due to overexpression of COX2 by macrophages and stromal cells. (*94*) High levels of HIF-α lead to inhibition of Phosphatase 2, which further leads to a prolonged activation of ERK/p38 signaling,because of insufficient dephosphorylation (*95*). This pathway is shown in figure 13.

HIF-α suppresses the expression of ER-α and enhances expression of ER-β by hypoxia at the transcriptional level. Also G-protein coupled receptor30 is also upregulated cy hypoxia in an HIF-1α-dependent manner, which also enhances the antiapoptotic effect of estrogen. (*95*) Hypoxia also induces angiogenesis in endometriosis. Different stimulation pathways lead to Angiogenesis, one of them is leptin signaling. Leptin upregulation through hypoxia directly induces production of VEGF-A. Since VEGF-A and Leptin are found aberrantly expressed in endometriotic cells, through transcriptional activation of HIF-α. (*96, 97*) Further Il-8 signaling is increased by the prolonged activation of ERK/p38, because of hypoxia induced downregulation of Phospholipase 2. This signaling cascade activates the CCAAT C/EBP α and β to bind to Il-8 promotor area. IL-8 further leads to angiogenesis. (*95*). Also another proangiogenic factor, which is less known, angiogenin is found upregulated in endometriotic cells. (*98*) Angiogenin is upregulated via an indirect signaling cascade induced by HIF-α, thus leading again to angiogenesis. (*95*)

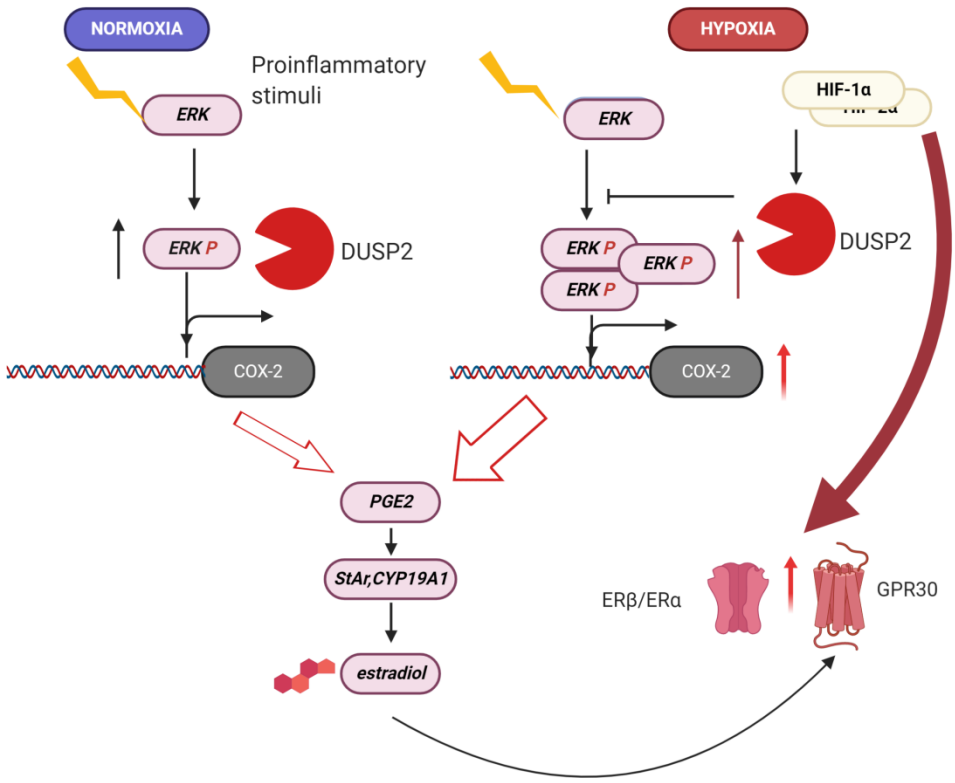


Figure 13 hypoxia in endometriosis modified from (95)

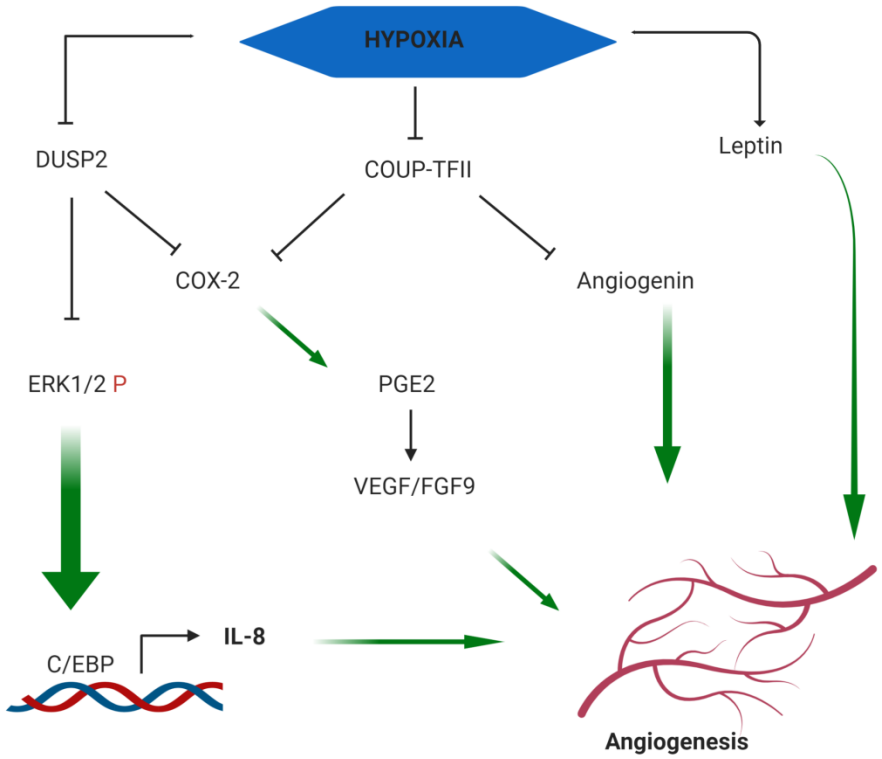


Figure 14 hypoxia induction of angiogenesis modified from (95)

Increased PGE2 concentrations also correlate with significantly high production of leukotrienes, which have been found in the peritoneal fluid of endometriosis patients (*99*) These eicosanoids are synthesized de novo and are mainly unstable. (*100*) However inducible

isoforms of COX and the PG synthase are undetectable under physiological conditions, but inflammatory activation by pro-inflammatory cytokines like Il-1β lead to increased induction of synthesis, especially secretory phospholipase A2 , COX-2 and microsomal prostaglandin E synthase-1. High Levels of 5-LO which leads to enhanced synthesis of leukotrienes and COX-2, Prostaglandin synthases, and secretory phospholipase A2 have been found in peritoneal macrophages and ectopic lesions. mRNA levels of 15-hydroxyprostaglandindehydrogenase was significantly downregulated in endometriosis patients compared to control. 15-PGDH normally inactivates PGE2 ,PGF2α and leukotrienes into inactive metabolites. (*101*)

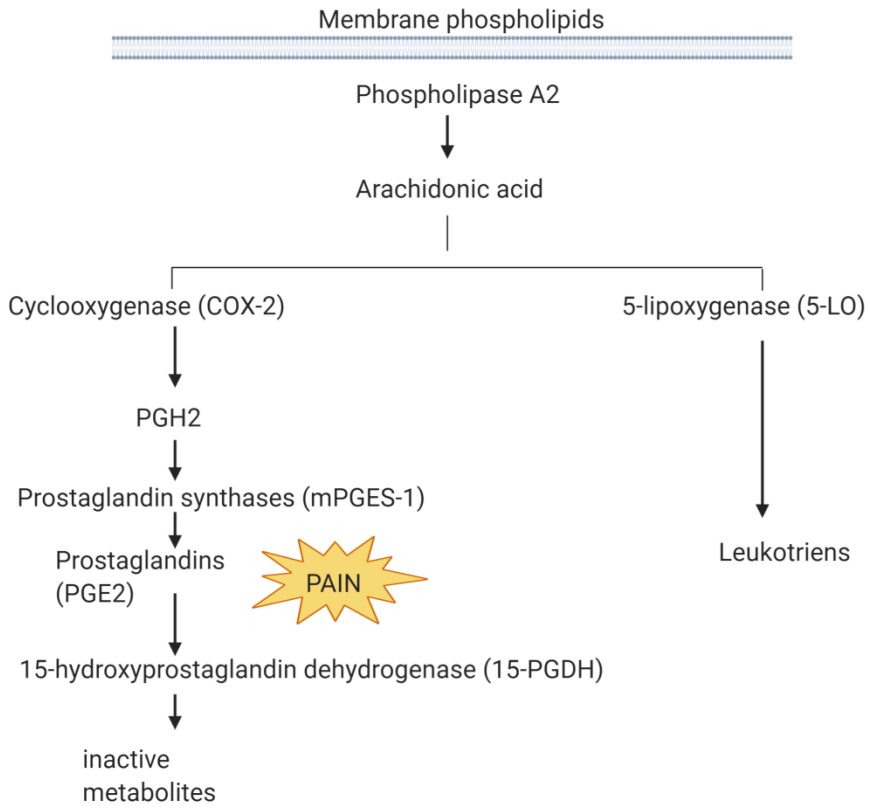


Figure 15 synthesis of prostaglandins and leukotriens in endometriosis modified from (101)

1.9 NF-κB signaling in endometriosis:

Signaling of NFκB as previously described plays a crucial factor in endometriosis development. One of the main interactions NF-κB has in endometriotic tissue is the communication with macrophages.

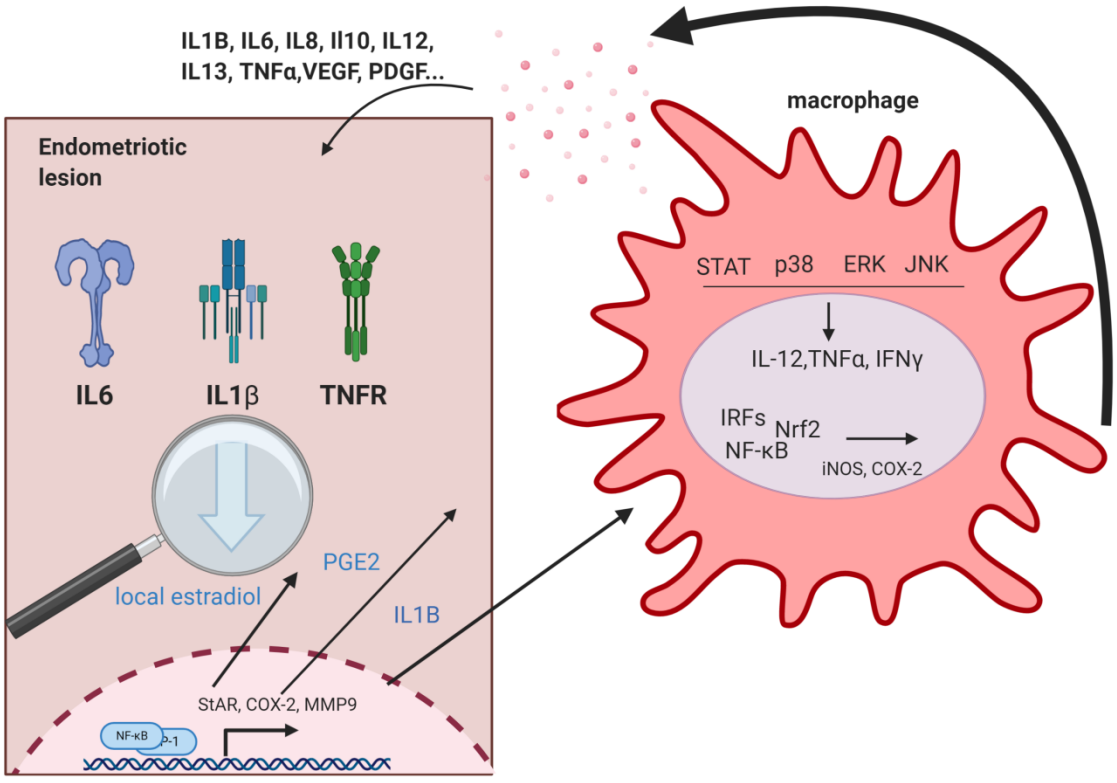


Figure 16 NFκB signaling between lesions and macrophages modified from (102)

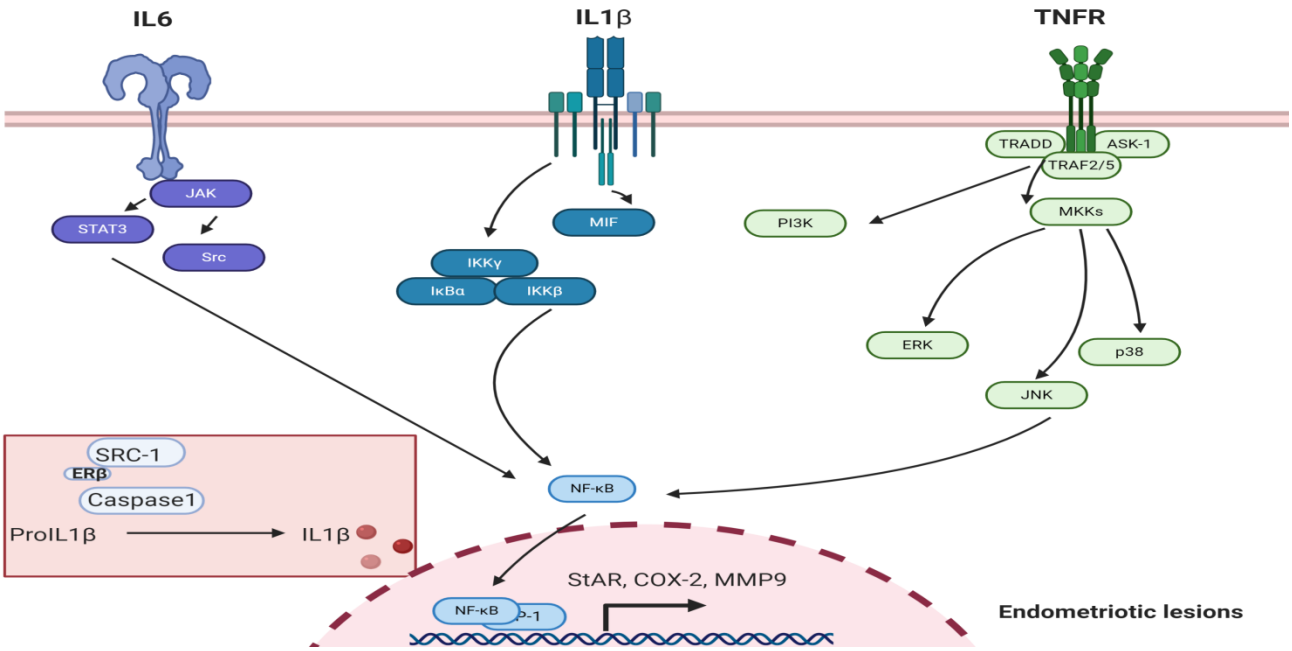


Figure 17 NFκB signaling inside the endometriotic lesions

Activation of the canonical NFκB Pathway leads to production of STaR, which is evolved in estrogen de novo synthesis in endometriotic cells, COX-2, which also further lead to estrogen synthesis and MMP-9. Again, the vicious circle of macrophages and endometriotic lesions is portrayed. Inflammation of stromal cells leads to high Il-1β production, which then activates macrophages , which produce more COX-2, more IL1β, more TNF-α leading to a constant inflammation process and constant NFκB activation in the endometriotic cells. (*102*) Constant NFκB signaling lead to angiogenesis, proliferation and antiapoptotic effects. An overview of NFκB signaling is shown in figure 19. The NFκB signaling can be activated via various pathways.

NFκB signaling is activated in normal endometrium during menstruation, when progesterone withdrawal kicks in. Endometriotic stromal cells are progesterone resistant, but NFκB signaling is constantly turned on. One of the main NFκB activators are TNF-α, Il1β and LPS, activating the canonical NFκB Pathway. NFκB inhibitors (IkB) are bound by the NFκB dimers. This complex is then phosphorylated by IKK the IkB kinase complex, further inducing proteasomal degradation. NFκB dimers are now free and activate gene transcription of their target genes. There are 3 different NFκB pathways. The canonical pathway is activated by Il-1β, TNFα and LPS. IKKβ is activated, thus p50/62 subunits are activated. The noncanonical pathway activates IKKα resulting in p52/RelB activation. The third NFκB signaling pathway is activated through different stimuli and activates dimers with various subunits. The non-canonical pathway is longer lasting than the canonical one and involves positive feedback through NFκB mediated synthesis of IL1β and TNFα activating the canonical pathway, which has a negative feedback by inhibition through IkBα, p100 and p105. NFκB signaling from endometriotic cells activates macrophages. The high iron concentration in the peritoneal fluid of endometriosis patients impairs phagocytosis and activates NFκB in macrophages thus resulting in more TNF-α and iL1β production. (*103, 104, 72*)

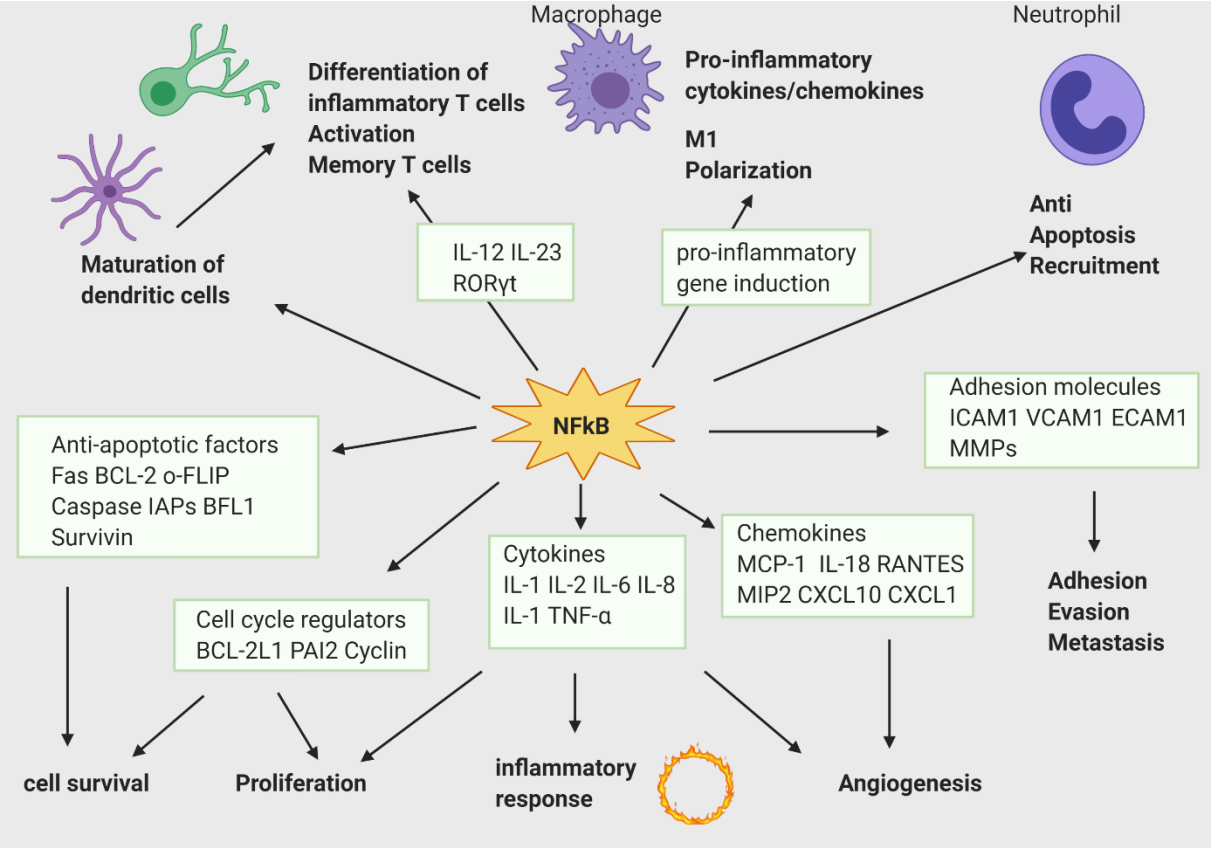


Figure 18 NFκB target genes modified from (105)

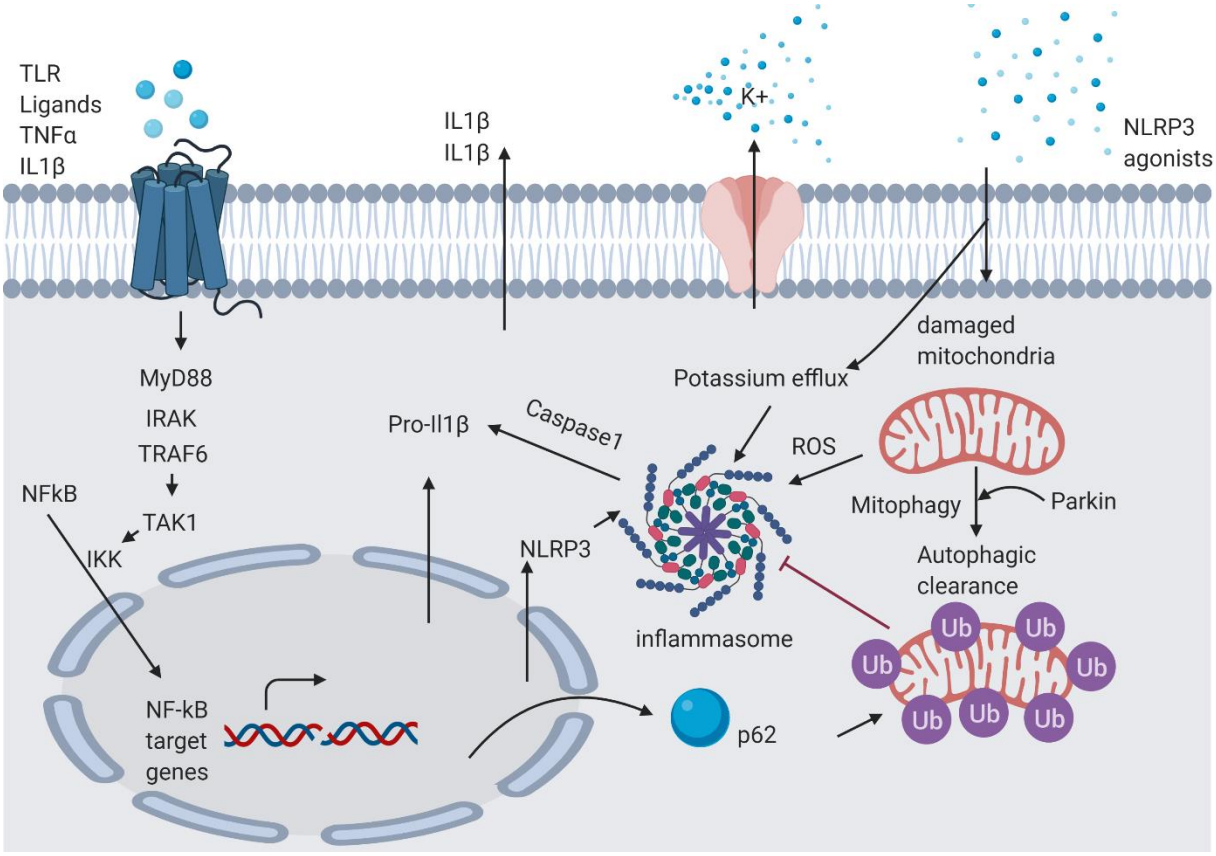


Figure 19 inflammatory NFκB response modified from (105)

1.9 Mitochondria in Endometriosis:

Mitochondria are involved in cell survival and energy metabolism, since they produce ATP and are initiators of intrinsic apoptosis pathway. Estrogen signaling also targets mitochondria, which express estrogen receptor (ERβ) on their membrane. An already used treatment for endometriosis is reducing estrogen to reduce mitochondria function. Since endometriotic cells must change their gene expression in a drastic way in order to be mobile and inhibit anoikic, a programmed cell death of epithelial cells, when they detach from the extracellular matrix, controlled mitophagy must happen in endometriosis. Mitochondrial fission is acquired in apoptosis and migration of cells. Zhao et. al found that Mst1 (Macrophage stimulating1) is downregulated in ectopic endometrial cells. (*105*) Mst1 is involved in tissue regeneration, adult stem cell proliferation and cancer development. (*106*) Also Mst1 has a regulating effect on p53 dependent mitophagy. Inactivation of Mst1 through overexpression of its transcriptional repressor leads to development of pancreatic cancer. Mst1 downregulation must have impact in development of endometriosis since re-introduction of Mst1 reduced endometriotic apoptosis. Mst1 activates phosphorylation activation of Drp1 and reduces Parkin gene expression via p53, which leads to mitochondrial fission and mitophagy inhibition. Zhao et al. proposed a mechanism shown in figure 21.

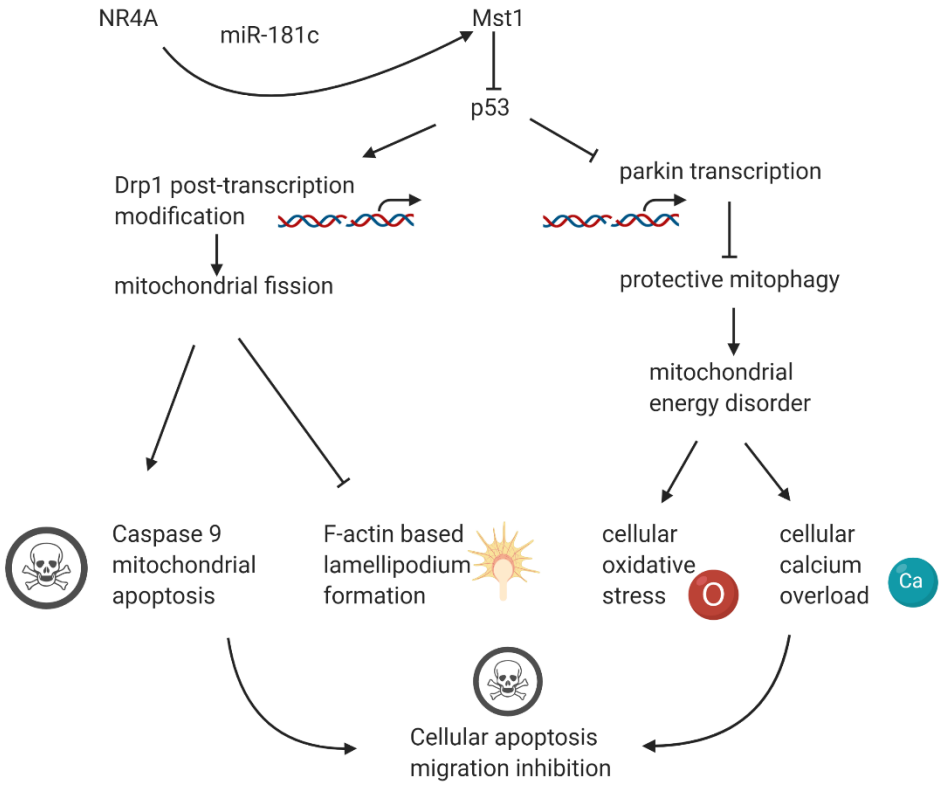


Figure 20 Mst1 signaling cascade modified from (105)

Downregulation of Mst1 leads to activation of parkin transcription and Drp1 phosphorylation activation. Mst1 activity leads to impairment of survival and mobilization of endometriotic cells. (*105*)

2. Proteomics:

Proteomics was first described in 1994 by Marc Wilkins, as the term "proteome" was established describing the protein complement of the genome. Therefore, proteomics encompassed the identification and quantification of the proteome , including isoforms, splicing variants and post translational modifications (PTMs) as well as protein expression and localization. (*107*)Proteomics is now a routinely used tool to detect and quantify thousands of proteins at once in a specific cell. Proteomic experiments can be performed in various approaches depending on the analytes. Top-down, middle-down or bottom-up approaches can be used. Top-down experiments are used for characterization of intact proteins, while middle-down and bottom-up proteomics focus on peptides for identification and quantification. Partially proteolytically digested peptides are used for middle-down proteomics, as these peptides are very large. (*107*) Bottom-up proteomics (also called shotgun proteomics) is used for protein samples derived from a tissue or cell lysate, which are enzymatically digested into short peptides using proteases like trypsin. Trypsin, a proteolytic enzyme, provides high specificity cleaving peptide bonds at the C-termini of arginine and lysine residues. (*108, 109*) Typically, liquid chromatography coupled to mass spectrometry (LC-MS) is used for bottom-up proteomic analysis. The peptide sample is separated using Liquid chromatography and elutes from the column directly in ionization source of the mass spectrometer. (*108, 109*) While mass spectrometry based proteomics, is now the state-of-the-art method, established proteomic techniques like SDS-PAGE and 2D gel electrophoresis methods are still used for specific scientific questions today. (*107*)

2.1 Bottum-up Proteomics/Shotgun Proteomics:

Quantification strategies can be divided into two main groups: label-based and label-free quantification (LFQ) methods. In label based proteomic analyses generally stable isotopes, which can be introduced chemically or enzymatically, are used. Chemical label-based proteomics techniques include ICAT(isotope-coded affinity tag),TMT (tandem mass tags) and iTRAQ (isobaric tags for relative and absolute quantification), while SILAC quantification is commonly used for metabolic labeling of amino acids in cell culture. (*110, 111*)

On the contrary label-free quantification is a semi-quantitative method in which peptides are not covalently modified. This enables a faster and simpler sample preparation and displays further higher dynamic ranges and proteome coverage. In Label-Free quantification approaches, different samples are running consecutively. Two approaches , based on MS1 and MS2 spectra can be used. In an MS1 spectrum, all intact peptides eluting at a given chromatographic time are visible. The height of each peak displays the number of detected tryptic ions, resulting in a so-called Intensity Score. Resolving power of current mass analyzers is high, still two peptides featuring the same amino acid sequence cannot be solely identified based on their intact masses. However, a characteristically fragment spectrum on the other hand, gives structural information of the compound. Concerning peptides, it is possible to fragment peptides at their weakest bonds using a collision gas. A so called MS2 spectrum

reveals the resulting fragment ions, which give clear information of the amino acid sequence, as well as post translational modifications. (*112*)

The MS1 signal for a given precursor ion is then integrated over time from all the MS1 spectra, where it is found. The area is a measure of the total number of ions for a particular peptide. It is more sufficient to use the area instead of the height. Still area quantification does not give the absolute number of peptides in the sample, but can be used for relative quantification. MS2 spectra are required for peptide identification but their signal is typically not used for quantification in these label-free approaches. For label-free quantification multiple runs are necessary, which reduces throughput. Also, low abundant proteins have a higher variability, leading to problems in the quantification. (*113, 114*)

Different peptides differ by their efficiency of their ionization. The magnitude of peptides in the ion current, deriving from a complex matrix, leads to an ion suppression. Also, efficiency of peptide analysis can vary due to digestion problems and peptide solubility. All in all, this would mean that the number of ions in the mass spectrometer is not a direct readout of how much of a protein was in the sample. Additionally, this means that quantitative mass spectrometry remains a challenge. (*115*)

Different bio-fluids like plasma, serum and urine to tissue samples and cell lines can be analyzed with bottom-up proteomics. Regardless of the sample type,a typical LFQ proteomic workflow always contains similar essential steps The sample is usually lysed using buffers , detergents and chaotropes in order to obtain solubilized protein samples. A Bradford assay or a so called bichinchoninic acid (BCA) assay is used to adjust the protein content is assessed, for the following digestions of the proteins. Digestion is generally carried out using trypsin. Proteins need to be incubated with ditiothreitol (DTT), where proteins are denatured. Reduction and solving of disulfide bonds takes place. After reduction proteins are alkylated using iodacteamid (IAA) in order to irreversibly block disulfide bonds from rearranging. Usually cysteines are alkylated. Proteolysis can be performed in solution, on filter or in gel. Peptides obtained are then subjected to the LC-MS. Usually, a reversed-phase chromatographic separation followed by high-resolution mass spectrometric analysis on TOF, FT-ICR or orbitrap instruments is used. To ensure reproducibility of the chromatographic run, stable-isotope labeled peptides are often added as internal standards. MS2 fragment spectra are used for Identification of the proteins. Deconvolution is performed. And spectra are searched against a theoretical peptide spectral database.

Deconvolution is needed to find the most likely peptide matches for the amino acid sequences. For this, theoretical MS2 spectra are generated for these peptides, based on all the b- and y-ions that can result from fragmentation. By comparison of the MS1 mass spectra and the corresponding observed MS2 spectrum of the theoretical spectra, the best match can be found, resulting in the peptide being assigned to the spectrum. Analysis are performed by various search algorithms, which perform the analysis automatically.

Improvement of MS- measurements has resulted in faster collection of MS2 spectra, covering more peaks from the MS1 spectrum. These recent developments might overcome missing value problems in the future. (*116*)

3. Mass spectrometry:

There a multiple LC-MS setups and different kind of mass spectrometers available. Since analysis in this thesis was performed with a Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer, this setup will be used for introduction into the topic. Analytes are being separated on a nanoflow HPLC column to reduce the complex matrix of the peptides. Nanospray ionization is used to ionize analytes into positive ion droplets. A capillary is used to transfer the analyte solution under high-voltage. The size of the droplets is continually reduced through evaporation of the solvent until the Rayleigh limit is reached. The surface tension of the droplets can no longer support the charge density and Coulomb explosion occurs. Smaller droplets with higher mass-to-charge ratio are created. After further solvent evaporation has occurred (multiply-)charged ions are being transported to the mass spectrometer through the transfer capillary(*117*) Electrospray ionization (ESI) can also be performed in "normal-flow" applications, but with proteomic analysis usually nano-flow and nanospray is used , in order to create smaller droplets, in which evaporation takes place much faster. In nanoflow applications, analysis takes longer time , which results in high analyte coverage, due to the low flow rate of LC system. (*118, 119*) The inlet of the Q Exactive orbitrap contains a transfer capillary, a heating block, an ion sweep cone, an S-lens and an exit lens. The geometry of the S-lens is an important improvement, which has been established over the last years, in order to optimize ion current. The ions are being transported through the capillary, which is surrounded by the heater block, creating temperatures op to 400°C. Pressure is decreasing, due to high vacuum creation inside of the MS, which is needed for an optimized ion transmission. Once the ions enter the S-lens region, Radio Frequency (RF) is applied to spaced electrodes, focusing and guiding ions towards the exit lens. The S-lens as well as the exit-lens are evacuated by a source vacuum pump. After the ions leave the S-lens region they are transported to the injection flatapole to a lower pressure region. The flatapole is used as an focusing appliance containing four electrodes. These electrons create an electric field alongside the electrode-axis. Hence the ions inside the flatapole are focused via high RF voltage and are further transported into the bent flatapole. The bent flatapole is used to guide ions in a 90° angle into the quadruple. Solvent droplets and neutral molecules are colliding with the walls of the flatapole and are removed. Ions are then focused into the quadrupole via the so-called TK (Turner-Kruger) lens. The first quadruple is used as a mass filtering appliance. A quadrupole is made of four round rods, arranged in a square symmetry. During injections, voltages on the rod-pairs are set to fixed voltage of opposite sings. Therefore, only specific ions with a specific mass-to-charge ratio pass the quadrupole. After leaving the quadrupole ions enter a gas-filled curved linear trap the so-called C-Trap. By entering the c-trap ions lose their kinetic energy colliding with inert gas, usually nitrogen. The C-trap is used as a collector of ions, where an automatic gain control (AGC) accuracy is performed. Collected

ions are extracted by increasing RF voltage on the C-trap rods. Therefore, ions are focused and accelerated as an ion beam into the orbitrap analyzer. An orbitrap mass analyzer is a highly sensitive analyzer, where Ion packets are oscillating around an inner electrode, through the high electric strength of the outer electrode. After all ions have entered the analyzer, an image current detection is performed, as the voltage is kept constant. The ion beam oscillates along the inner electrode, their frequency dependent on their respective mass/charge ratio.

An image current is created by the oscillating ions through slits in the outer electrode. This signal is amplified and converted from analog to digital. Fourier Transformation is used to transform the signal into m/z ratios. For the MS2 spectra, the ions are passed on into the higher energy collisional dissociation (HCD) cell. The HCD contains collision gas, creating ion fragments. Ions collected in the C-trap are transported into the collision cell using the collision gas. The ion fragments are transferred again into the C-trap, where they wait to be injected into the orbitrap analyzer. This application is used to created tandem mass spectrometry. (*120*) (*121*)

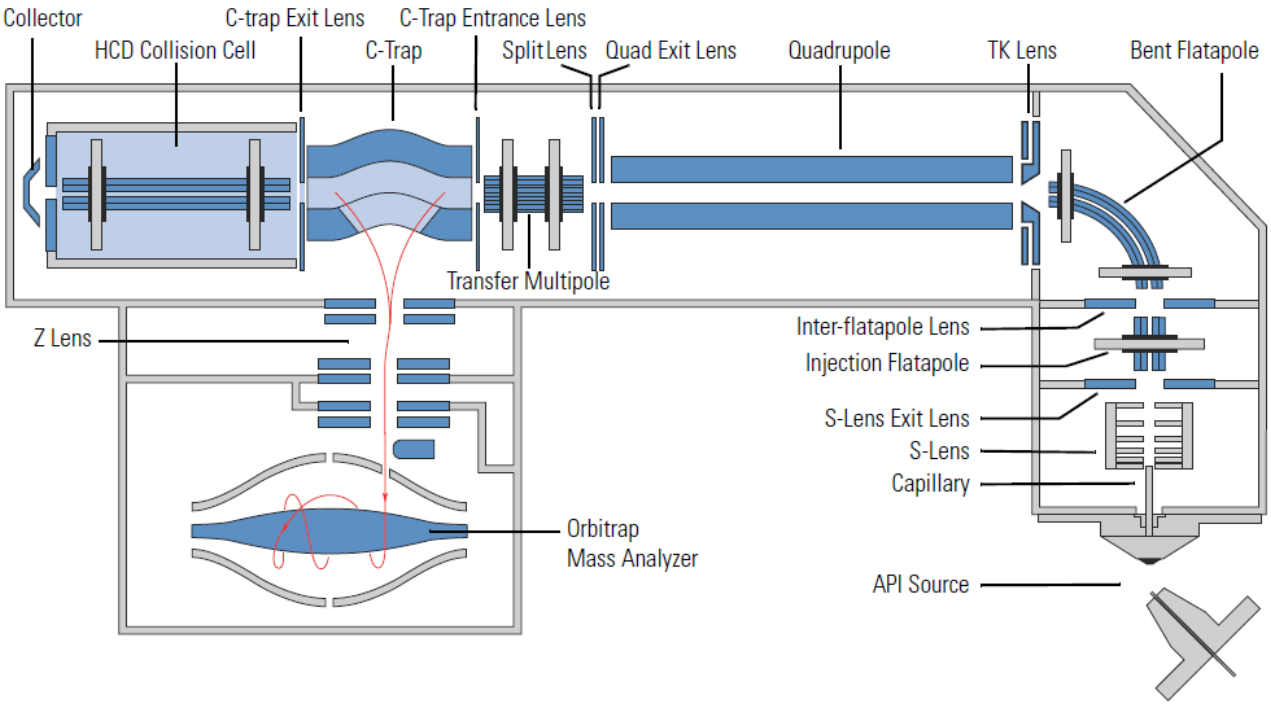


Figure 21 schematic of the Q Exactive taken from (*120*) all rights to Thermo Scientific

4. Objectives:

The aim of this master's thesis was to give insight into the proteomic differences of endometriotic epithelial cell line 12-Z and endometrial stromal cell line THESC upon inflammatory stimulation. Label free proteomic approaches were used for profiling of the inflammatory response in endometriotic cells. As the pathogenesis of endometriosis still remains unclear, high performance techniques might be able to give further valuable

information. Endometriosis patients have altered response to inflammatory signaling. Therefore, assessment of proteomic alterations in response signaling should give deeper insight into differences. Endometriotic environment consist of both stromal and epithelial cells. The immune inflammatory interactions of these cells are stimulated by an autocrine and paracrine way, mostly via IL-1β and TNFα, secreted by macrophages. Therefore, approaching a cell culture model of inflammatory reactions could give specific endometriotic alterations. Since proteomic approaches of cell culture of endometriotic cells have not been well described before, this thesis should open new horizons concerning research approaches in endometriosis studies. Hence, the thesis will focus on molecular profiling of endometriotic epithelial and endometrial stromal cells cultivated in in-vitro conditions. Healthy stromal fibroblasts cell line might display proteomic alterations in an in-vitro inflammatory study, compared to already chronically inflamed endometriotic cells. Since the 12-Z cell line, is one of the only cell lines available for endometriosis studies. This thesis should gain deeper understanding of the alterations underlying this endometriotic cell line compared to in vitro inflamed healthy fibroblast cells. Comparison to artificially inflamed non-endometriosis endometrial cell line 'THESC' gives further insight into the profile of 12-Z endometriosis derived cell line. High resolution approaches in profiling in-vitro analysis of inflammatory stimulated endometrial fibroblasts, the persistence and continuous progression of this chronic inflammatory disease was assessed-

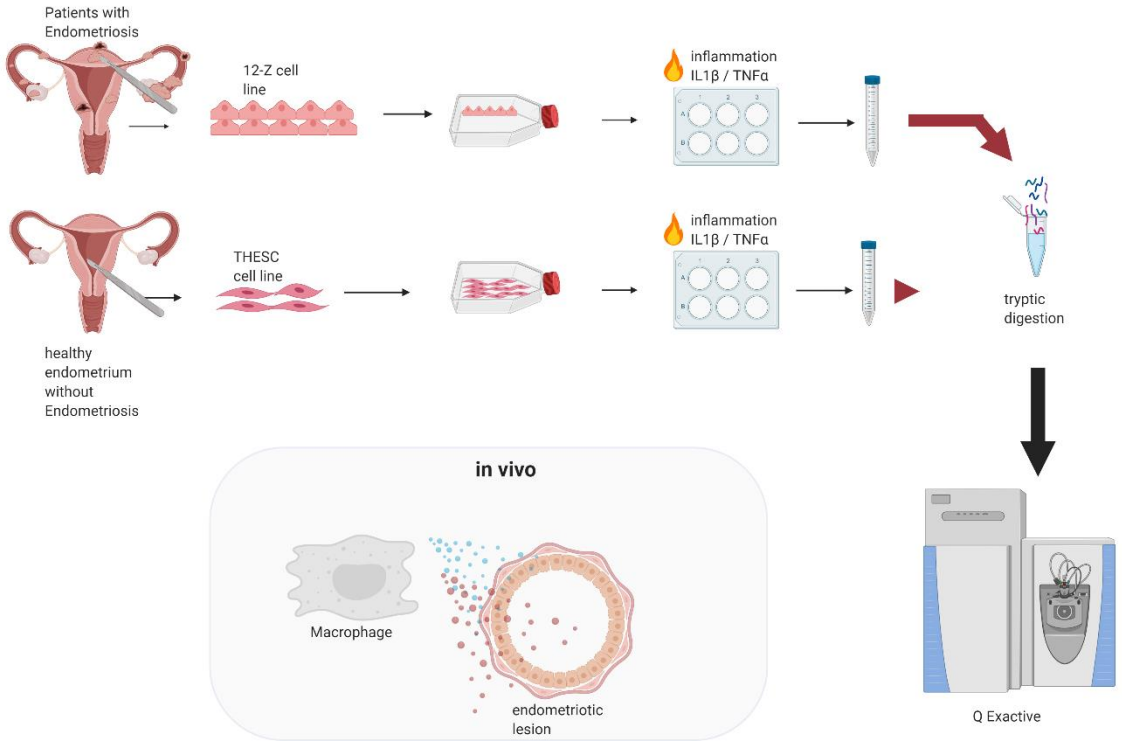


Figure 22 Master Thesis workflow; celllines are created from tissue derived cells, epithelial endometriotic cell line and endometrial stromal cell line thesc, cultivation and inflammatory stimulation with IL-1β or TNF-α preparation for mass spectrometry based proteomic

5. Material and Methods:

5.1 Cell collection and acknowledgments:

Already processed primary cells and human endometrial cell line THESC were graciously obtained by the Medical University of Vienna. The 12-Z cell line was a generous gift of Dr. Anna Starzinski-Powitz (Goethe-Universität Frankfurt) and were isolated under approval by the local ethical committee.

5.2 Cell culture and sample collection:

Human endometrial cell line THESC was cultivated in DMEM-F12 phenolredfree (Gibco, Thermo Fisher Scientific, Austria) with 10% (v/v) heat inactivated Fetal Calf Serum (FCS, Sigma-Aldrich, Austria) and 1% (v/v) Penicillin and Streptomycin (Sigma-Aldrich, Austria). Cultivation was done in humidified incubators at 37°C and 5% CO2 (Heracell 150i C02 incubator, Thermo Fisher Scientific, Austria). THESC fibroblastic endometrial cell line was grown in T75 polystyrene cell culture flasks with cell growth surface for adherent cells (Starstedt, Austria). Cells were sub-cultured every 5 days. After removing the medium, the cells were washed with 2 ml PBS and incubated with 3 ml Trypsin-EDTA solution 0.25% (Sigma Aldrich, Austria) for 4 minutes. After incubation, the reaction was stopped by adding 4 ml fully supplemented medium into the flask. The cell suspension was then centrifuged for 5 min at 220xg. The remaining supernatant was removed and cells were resuspended fully supplemented medium and split at a 1:3 ratio. Therefore, the cells were resuspended in 3ml fully supplemented medium and 1 ml was transferred into the T75 culture flask, containing 9ml of fresh preheated Medium (37°C). Cells were cultivated in 6 T75 flasks until they had reached a concentration of 5 million cells/ml. Cell counting was performed using a MOXI Z Mini Automated Cell Counter (ORFLO Technologies, USA) using Moxi Z Type M Cassettes (ORFLO Technologies, USA). Inflammatory treatment cell concentration was set to 100.000 cells/ml and cells were seeded in replicates, in 3ml fully supplemented medium in a 6-well plate with cell growth surface for adherent cells (Starstedt, Austria) at a density of 300.000 cells/well.

Culture conditions were similar for 12-Z cell line culture. 12-Z cells were thawed and transferred into 10ml fresh preheated fully supplemented medium DMEM-F12 phenolred-free (Gibco, Thermo Fisher Scientific, Austria) with 10% (v/v) heat inactivated Fetal Calf Serum and 1% (v/v) Penicillin and Streptomycin. The cell suspension was centrifuged for 5 min at 220g and supernatant was discarded. Cells were resuspended in fully supplemented medium and cultivated as with THESC cell line conditions. 12-Z cell lines were sub-cultured every 2-days as their growth rate was very high. All cells were routinely checked for mycoplasma using CASY TT Cell Counter and Analyzer (OMNI Life Science GmbH & Co. KG, Bremen, Germany).

5.2.1 THESC sample collection:

5.2.1.2 Proteomics:

Cells were inflammatory activated at different time points. After cultivation for 24 hours in 6 well plates inflammatory stimulation was applied for 48h hours at a concentration of 10ng/ml for the respective replicates. After incubation, supernatant was discarded, the cells were

washed twice with PBS and fractionation was performed. (*122, 123*) 1 ml of isotonic lysis buffer (10mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10mM NaCl, 3mM $MgCl_2$, 0.5% Triton X-100, Protease and Phosphatase Inhibitor cocktail (Sigma-Aldrich)) was added to the cells , while cells were scrapped using a cell scraper. Cell lysis was achieved using mechanical shear stress using a syringe and a needle. Cell suspension was pressed out of the syringe and pressed against the wall of the Falcon tube for 15 times. An aliquot was checked under a light microscope whether the cells were lysed. The cell suspension was centrifuged at 2270g for 5 minutes. Supernatant containing cytoplasmic fraction was precipitated in 4 ml of EtOH. The remaining pellet containing nuclear fraction was discharged, due to too low protein concentration, which was assessed during preliminary studies (data not shown).

5.2.2 12-Z sample collection:

5.2.2.1 Proteomics:

After supernatant was removed, all steps prior described for THESC were taken on for proteomic collection of 12-Z cytoplasmic samples.

5.3 Sample preparation:

5.3.1 Proteomics:

5.3.1.1 Protein concentration determination BCA Assay:

After precipitation, cell suspension was centrifuged at 4536g for 30 minutes (4°C). Supernatant was discarded, while pellet, containing proteins, was dried under vacuum for 20 minutes. After the cell pellet had dried, it was resuspended in lysis buffer (8M urea, 50mM TEAB, 5% SDS). For the supernatants 70 µl were used, while for cytoplasmic fraction 50µl of lysis buffer was used. Protein concentration was determined with usage of a bicinchoninic acid assay. For this reagent A (26mM bicinchoninic acid disodium salt hydrate, 186 mM sodium carbonate, 8mM sodium tartrate, 113mM sodium bicarbonate, pH 11.25) and reagent B (200mM copper sulfate pentahydrate) were prepared. Then reagent A and reagent B were mixed at a 50:1 ratio for preparation of the working reagent. A 96-well plate (Starsted, Austria) was used for determination of protein concentration. Therefore, a calibration curve using a BSA (1µg/µl) standard was pipetted. Therefore, 200µl of working reagent were mixed with 0 to 8 µl of the 1 µg/µl BSA standard and 1µl of lysis buffer adding the respective amount of LC-MS grade water (VWR, international, Austria). 1µl of the protein sample was loaded to 9µl of LC-MS grad water and 200µl of working reagent. The 96-well plate containing calibration curve and protein sample solutions was incubated at 60°C in the dark for 30 minutes. After incubation, samples were mixed slowly, remaining in the 96-well plate, and absorptions were measured at a wavelength of 562 nm.

5.3.1.2 Protein digestion protocol using Protifi S-Trap

After determining protein concentration according to BCA protocol. An aliquot of the samples containing 20µg of protein was taken for protifi digestion. The samples were then diluted to a concentration of 1µg/µl. 20µl of the diluted sample (20µg) were pipetted into a 1,5 ml Eppendorf microcentrifuge tube and 20µl of 64mM DTT was added, for reduction of disulfide

bonds. Samples were heated for 10 minutes at 95°C at slow agitation (300 rpm). The sample was cooled to room temperature and then cysteines were alkylated using the respective amount of 5µl of I 486 mM IAA solution. Slight agitation (300 rpm) was used to incubate the samples with the IAA in the dark for 30 minutes at 30°C. Afterwards, 4.5 µl of 12% phosphoric acid was added resulting in 1,2% final concentration of phosphoric acid. 297µl of S-Trap buffer (90%MeOH (v/v), 0,1 M TEAB) were added to the solution. Then the sample was loaded on the Protifi digestions column, which was placed in a 2 ml Eppendorf microcentrifuge tube. The lysate was loaded two times with 175 µl respectively and a centrifugation step at 4000 g for 1 min in between the loading. Afterwards, the column was washed 4 times using the respective amount of 150µl of S-trap buffer The column was then transferred in to a new Eppendorf microcentrifuge tube and Trypsin/LysC (MS grade; Promega Corporation, Madison, USA), dissolved in a 1:40 ratio in 50mM TEAB, was added for the respective µg of protein, hence 20µl of enzyme was pipetted into the solution. Protein samples were incubated for 2 hours at 37°C in a thermomixer. After incubation, the digested peptides were eluted. Therefore, 40µl of 50mM TEAB were directly loaded onto the digestion solution. After a 1 min centrifugation step at 4000xg, 40 µl of 0,2 % formic acid was added. Samples were again centrifuged for 1 minute at 4000xg, before adding 35µl of 50% ACN/0,2% formic acid. After a 1-minute centrifugation the samples were dried using a vacuum centrifuge.

5.4 Mass spectrometry and LC-method:

5.4.1 Proteomics:

The peptide pellet was reconstituted in 5µl 30% formic acid containing a mixture of synthetically produced standard peptides (10 fool/µl) and diluted with 40µl of mobile phase A (97,9% H20, 2% acetonitrile, 0,1% formic acid). The sample solution was then transferred into a 96-well plate and placed into the autosampler (4°C) of the HPLC (Dionex UlitMate 3000 RSLCnano) coupled to QExactive Orbitrap MS (all Thermo Fisher Scientific, Austria). For concentration and trapping of the sample, the sample was transferred to a pre-column (Acclaim Pepmap 100, 100µm x 2 cm, nanoViper; C18, 5µm, 100 Å) at a flow-rate of 10µl per minute of 100% eluent A (97.9% H2O, 2% acetonitrile, 0.1% formic acid).The sample was further transferred onto the 50 cm × 75 µm Pepmap100 separation column (Thermo Scientific, Austria) and separated at a flow rate of 300nL/min. Cytoplasmic fraction samples were eluted using the following 95 min gradient from 8% to 40% mobile phase B.(79.9% acetonitrile, 20% H2O, 0.1% formic acid).Therefore 1% B for 9 minutes, 1-8% B for 1 minute, 8-30% B for 80 minutes, 30-40% B for 5 minutes, 5 minutes hold at 80% B, until decreasing the percentage to 1% B for 3 minutes, followed by 17 minutes equilibration time at 1% B. The total LC method time was 135 minutes.

For Masspectrometry setting were the same for all samples. The MS1 resolution level was set to 70,000 (at m/z=200) with a scan range from 400 to 1,400 m/z. A top eight method (using the top eight abundant peptide ions) was chosen for fragmentation at 30% collision energy.

Created fragments were analyzed in the Orbitrap mass analyzer at a resolution of 17,500 (at m/z=200). Chromatographic peak width was set to 10 seconds. The whole duration time was 115 min. A detailed table of parameters is shown in table 1.

Table 1 detailed proteomic Masspectrometry method

PARAMETERS	WHOLE CELL LYSATE
METHOD DURATION	115 min
MS1 RESOLUTION	70,000
MS1 AGC TARGET	$3e^6$
MS1 SCAN RANGE	400-1400 m/z
MS2 SCAN RESOLUTION	17,500
MS2 AGC TARGET	$2e^4$
TOP N	8
DYNAMIC EXCLUSION [S]	45s
CHROMATOGRAPHIC PEAK WIDTH [S]	10 s

5.5 Data analysis:

5.5.1 Proteomics:

Raw data, obtained from the mass spectrometer was submitted to the freely available software MaxQuant (version 1.6.6.0) utilizing the Andromeda search engine, to obtain LFQ values. All measurements were searched against a Uniprot derived fasta file (the human Uniprot database (version 03/2018, reviewed entries only) with 20,316 protein entries) of human proteins. A minimum of two peptide identifications, at least one of them being a unique peptide, were needed for protein identification. Variable modifications included in the analysis was carbamidomehtlytion of cysteine and. oxidation of methionine and acetylation of the protein C terminus. Digestion mode was set to Specific including digestion enzyme Trypsin/P. A maximum of two missed cleavages was accepted. FDR for peptide spectrum match as well as protein FDR was set to 0.01.

Data was further processed for statistical analysis in software package Perseus (version 1.6.6.0) Filtering for proteins only identified by site, as well as reverse identifications and potential contaminates was performed. For further statistical evaluation missing values were filled up and the LFQ intensities were log2 transformed. Samples were grouped according to their respective replicates and the minimum number of valid values was set to two in at least one group. PCA and Volcano Plots were created, identifying the significantly regulated proteins. Significantly regulated proteins were further analyzed using the Cytoscape plugin Clue Go (using standard default settings). Resulting in network analysis based on gene ontology (GO). Furthermore, String analysis were performed in order to identify most active transcription factors, which have not been found due to low abundance.

6. Results:

6.1 Proteomic analysis of the 12-Z epithelial endometriotic cell line

. Proteomic analysis of the 12-Z cells both with TNFα and IL1β stimulation resulted in total number of 4250 proteins identified applying a false discovery rate of 1%. A Principal component analysis (shown in figure 23) shows a separation of the samples upon their different respective inflammatory activation. Inflammatory stimulation with Il1β is shown in red, while TNFα stimulation is colored in blue. Both inflammatory activated batches are separated from control group shown in green.

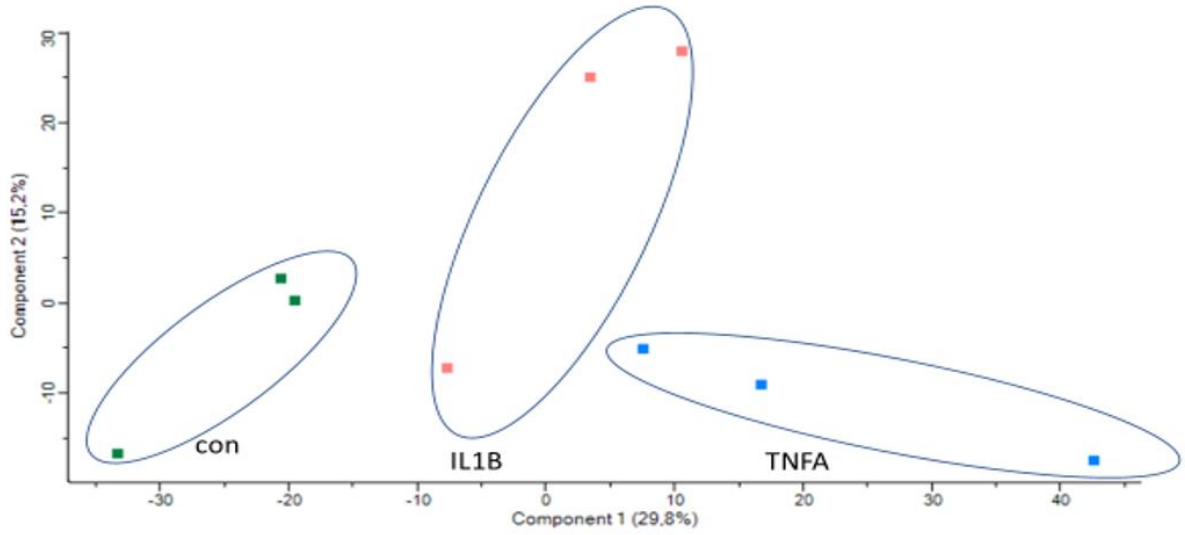


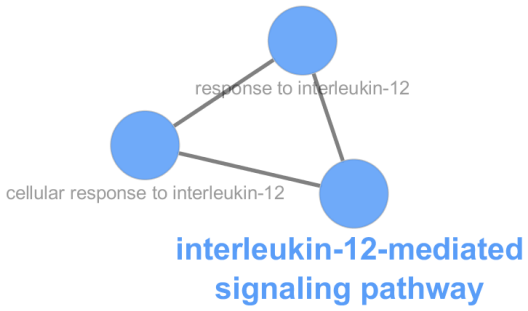
Figure 23 Principal component analysis of 12-Z cells

Stimulation with IL1β and TNFα resulted in different responses to inflammation compared to the control (non-treated) group, which is already chronically inflamed due to its endometriosis state. Stimulation with TNFα resulted in more regulated proteins (510) than IL1β stimulation (118 regulated proteins). Generally, the analysis of inflammatory activated and endometriosis cells without treatment showed different alterations in the proteome profile such as angiogenesis and EMT. Also, proteins essential for cell-neuron interactions are found to be upregulated upon stimulation with the respective inflammatory agents. Overall the proteomic profiles created upon different stimulations is reflected in the observed data

6.1.2 General proteome alterations upon inflammatory stimulation

In both IL1β stimulation as well as TNFα stimulation classical inflammatory signature was found in the proteomic profile. Proteins found upregulated involved in inflammatory response (e.g. **SERPBINB2**, **SOD2**, **ICAM1**, **S100A8**) reflected IL-12 signaling upon NFκB signaling pathway activation. According to Cytoscape analysis, Interleukin 12 mediated signaling pathway was found upregulated upon inflammatory stimulation. Also, different interleukin signaling responses were found according to the respective stimulation. Activation with TNFα resulted in IL-6 pathway upregulation, while IL1β resulted in response activation of Interleukin

4 and secretion of IL-8. Overall, both stimulations induced a pronounced inflammatory signaling reflected in the proteome alterations.



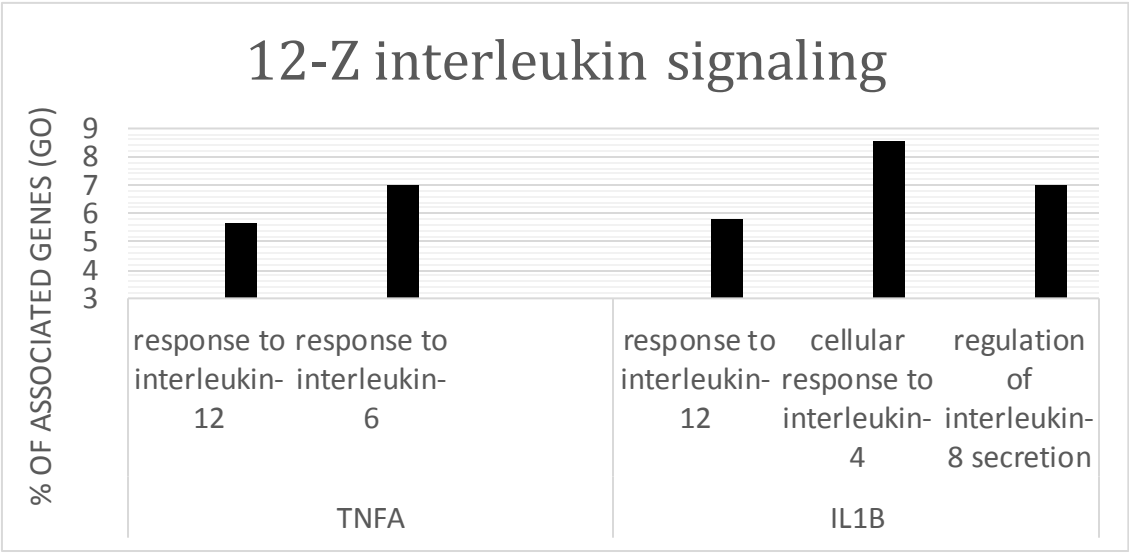


Figure 24 Cytoscape analysis showing upregulation of Interleukin signaling pathway

6.1.3 Stimulation with IL1β

Inflammatory stimulation with Interleukin-1β resulted in the statistically significant regulation of 118 Proteins. Figure 25 shows a general overview on proteins regulated portrayed in a volcano plot. The right side of the volcano plot displays upregulated proteins, while the left side portrays downregulation of the proteins. Up or downregulation correspond to higher or lower LFQ Intensities, respectively.

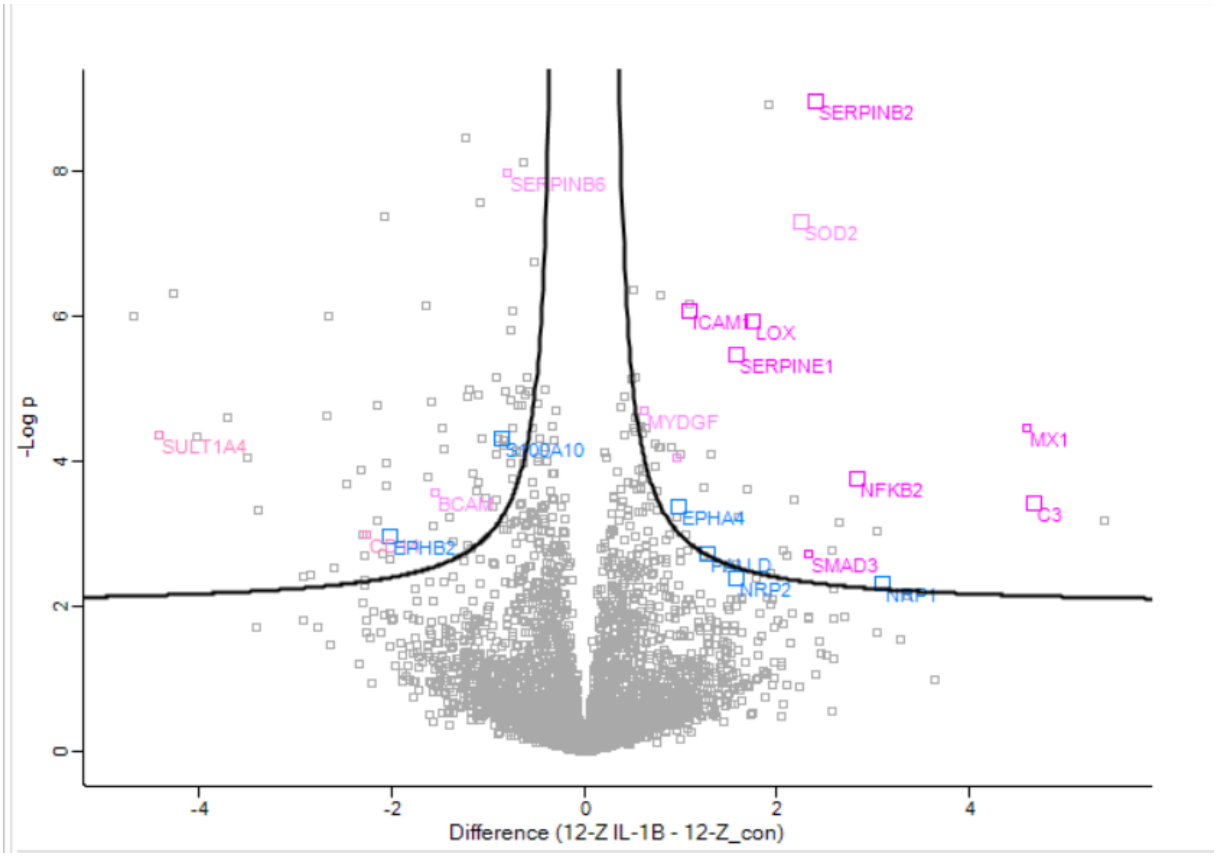


Figure 25 volcano plot showing alterations in relative protein abundance between inflamed and non-treated control; FDR = 0.05, S0=0.1

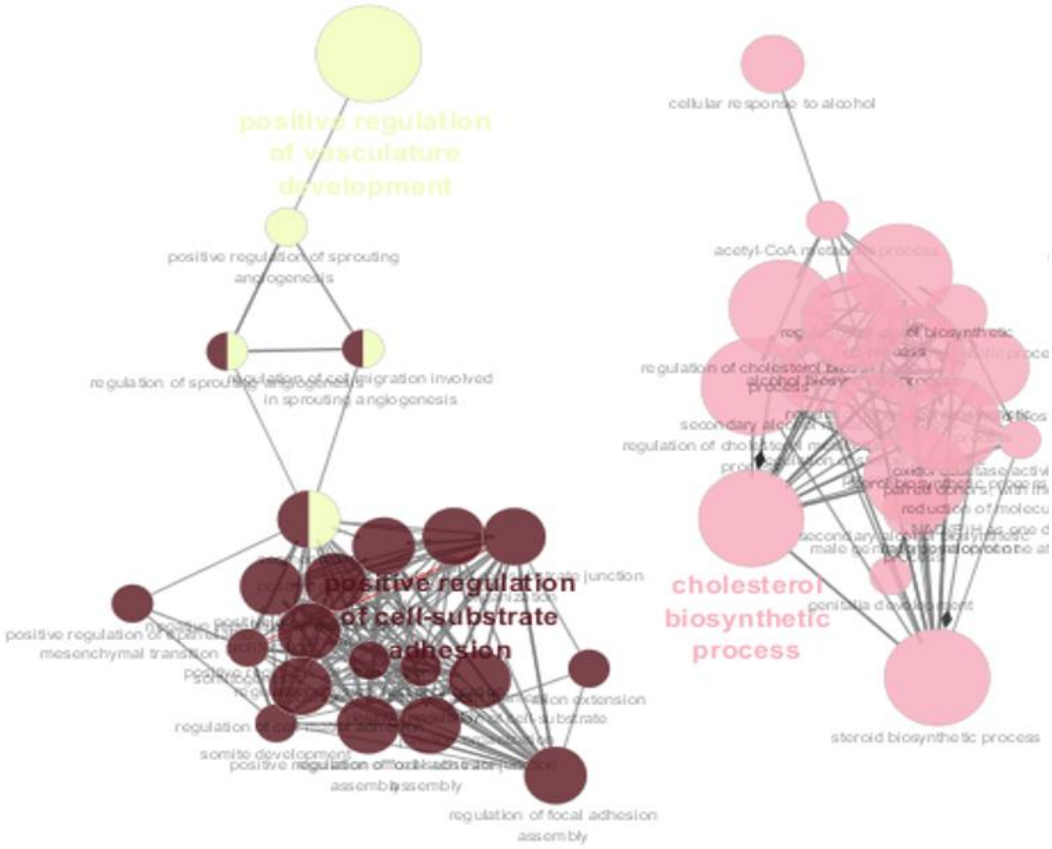


Figure 26 biological processes regulated during stimulation

Most abundant pathway cluster regulated are involved in angiogenesis and EMT, as well as regulation of cholesterol biosynthetic processes. Upregulated proteins were mostly involved in angiogenesis and vascularization, neuronal recognition as well as metabolic processes. Downregulated proteins were involved in cholesterol biosynthetic processes, steroid synthetic processes and apoptosis.

6.1.3.1 Cholesterol biosynthesis

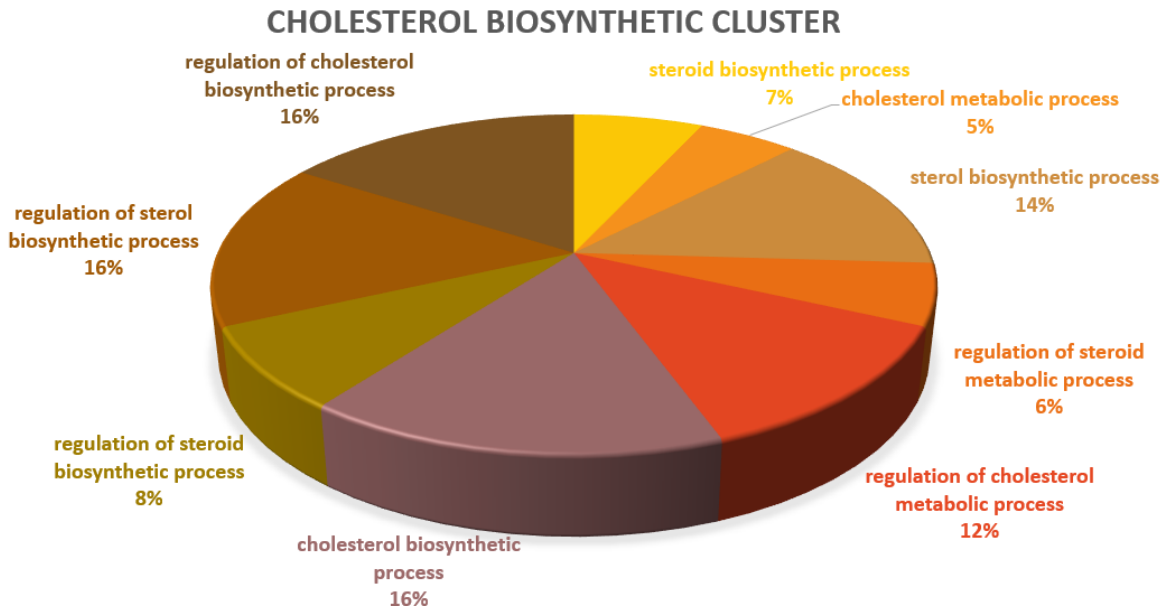
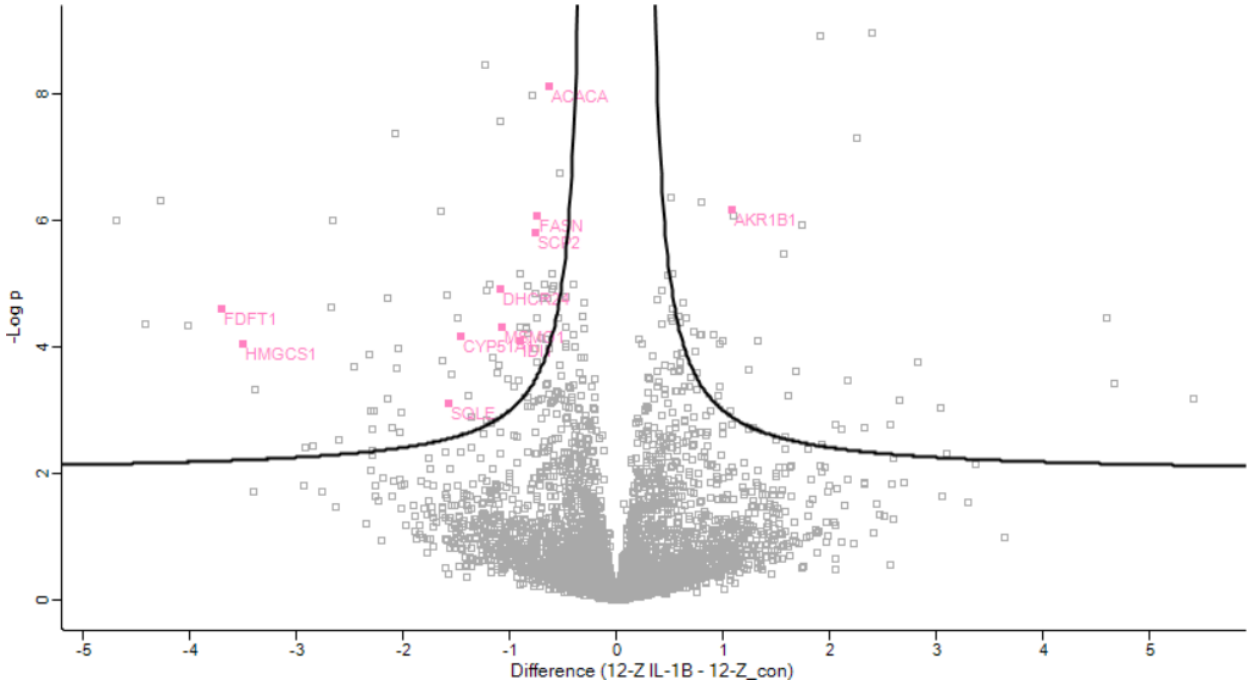


Figure 27 pathways regulated within cholesterol cluster (% of associated genes according to GO)

The proteomic analysis of the inflammatory activated endometriotic cells showed a reduction in proteins important for synthesis of cholesterol and steroids. Pathways within the biosynthetic cluster regulating sterol biosynthetic processes were found abundantly regulated. Contrary to expectations from literature (2, 40, 30) the stimulation with IL1β resulted in a downregulation of proteins involved in cholesterol pathway, as shown in figures 27- 29

Figure 28 volcano plot showing downregulated proteins involved in cholesterol biosynthetic processes; FDR=0.05 S0=0.1,



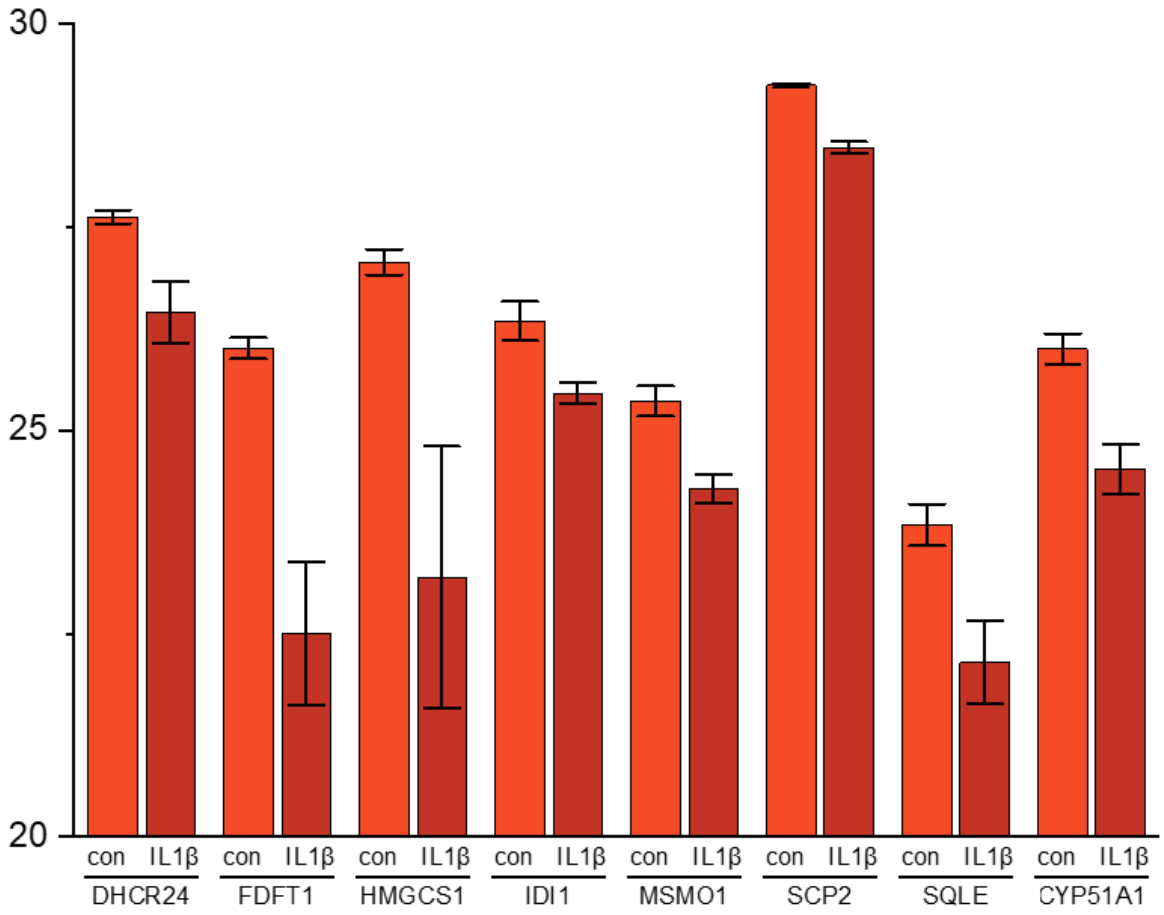


Figure 29 Proteins downregulated involved in cholesterol biosynthesis

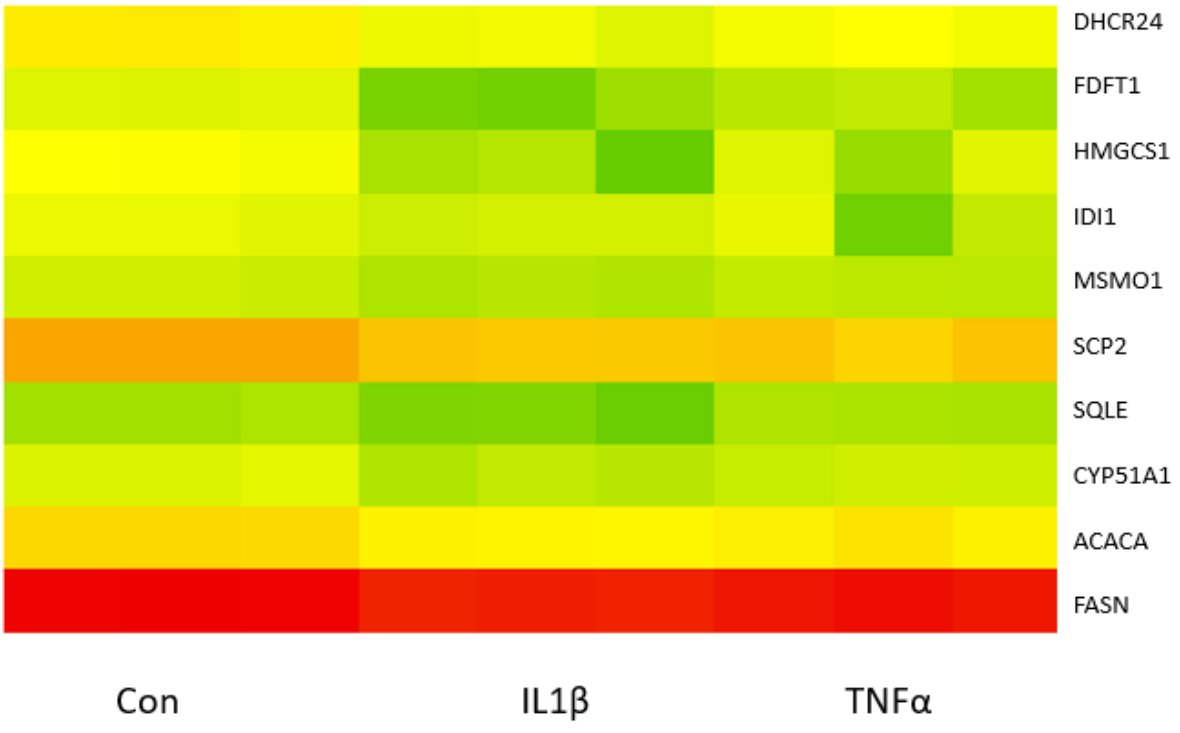


Figure 30 heatmap showing altered protein expression involved in cholesterol biosynthesis upon IL1ß and TNFα stimulation

Figure 30 shows alterations in protein expression levels upon stimulation with the respective inflammatory agents. The expression levels correspond to higher or lower LFQ Intensities of the proteins. Proteins involved in biosynthesis are mostly enzymes such as **FASN**, **SQLE**, **FDFT1** and **HMGCS1**. It was noticeable that all proteins involved in biosynthesis of cholesterol have lower LFQ Intensities upon stimulation with IL1β than control group (hereafter referred as con) and also TNFα stimulation. LFQ Intensities are still high but go down upon inflammatory stimulation. TNFα stimulation also results in lower LFQ Intensities of the enzymes, but without any significant regulation. This finding can also be observed with FASN. Fatty acid synthase is highly expressed in control group as seen with dark red color. The TNFα group has a slightly darker color than IL1β. Generally, endometriotic epithelial cells (without further stimulation) display higher LFQ values, which might be due to their already endometriosis state.

6.1.3.2 Neuron recognition

Proteins involved in neuron recognition and axonal growth signaling were found upregulated upon Il1β stimulation. These proteins are commonly secreted by cells for axonal guidance needed for neuronal growth during development.

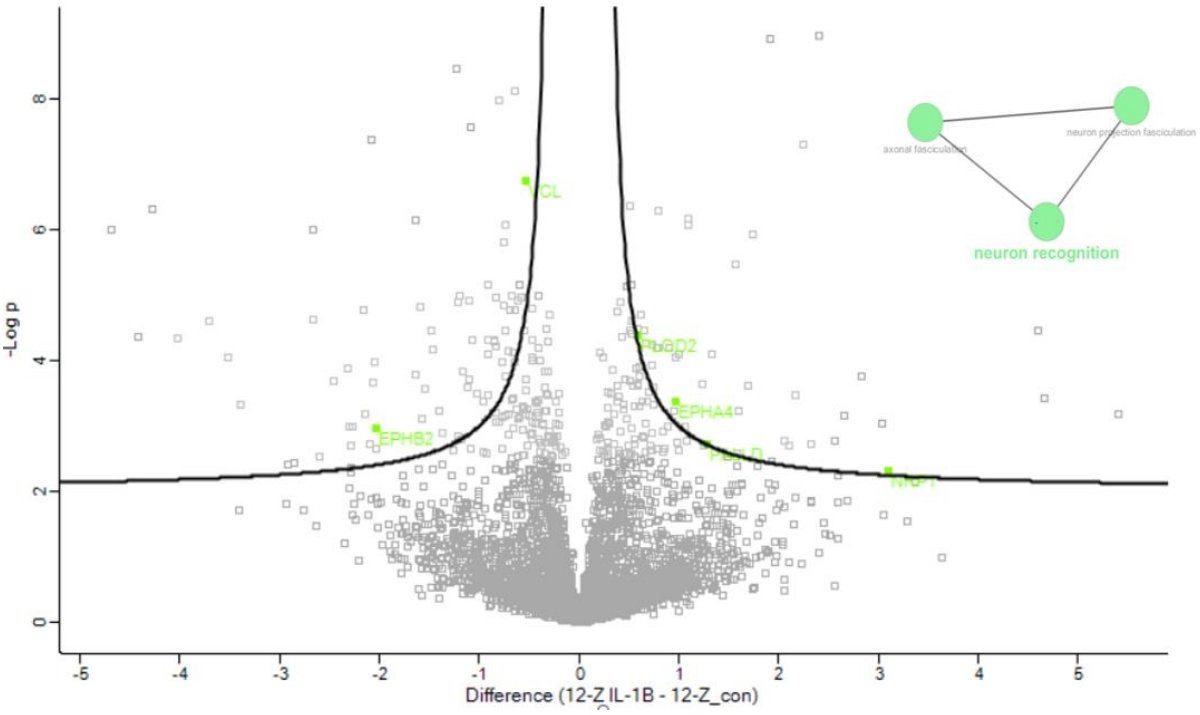


Figure 31 Cytoscape analysis of pathways identified in neuron recognition ; volcano plot showing proteins involved in axonal guidance; FDR=0.5; S0=0.1

NRP1, **PLOD2, EPHA4** and **PALLD** were found upregulated, while **VCL** and **EPHB2** were found significantly downregulated, compared to non-treated control group (Figure 31).

6.1.3.3 Angiogenesis and EMT

Generally, stimulation with IL1β resulted in upregulation of proteins characteristic for angiogenesis and epithelial to mesenchymal transition, according to Cytoscape analysis.

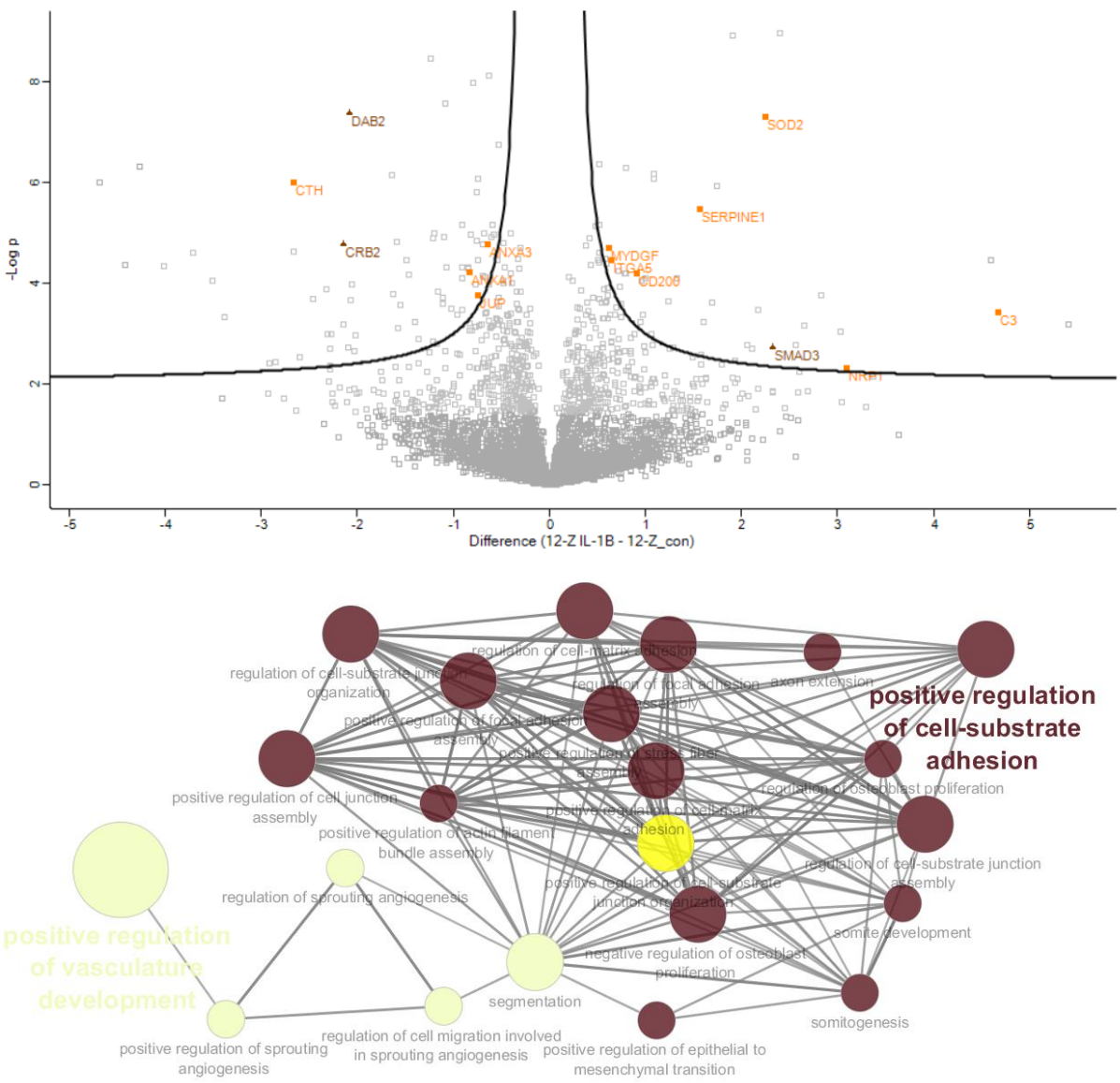


Figure 32 cytoscape analysis of angiogenesis ; volcano plot showing regulated proteins involved in angiogenesis and EMT; FDR=0.5,S0=0.1

Proteins found upregulated are mostly involved in positive regulation of vasculature development, regulation of sprouting angiogenesis and regulation of cell migration involved in sprouting angiogenesis. Proteins found significantly upregulated like **ANXA3** and **ANAX1** are angiogenic factors, increasing endothelial cell migration. (*124*)

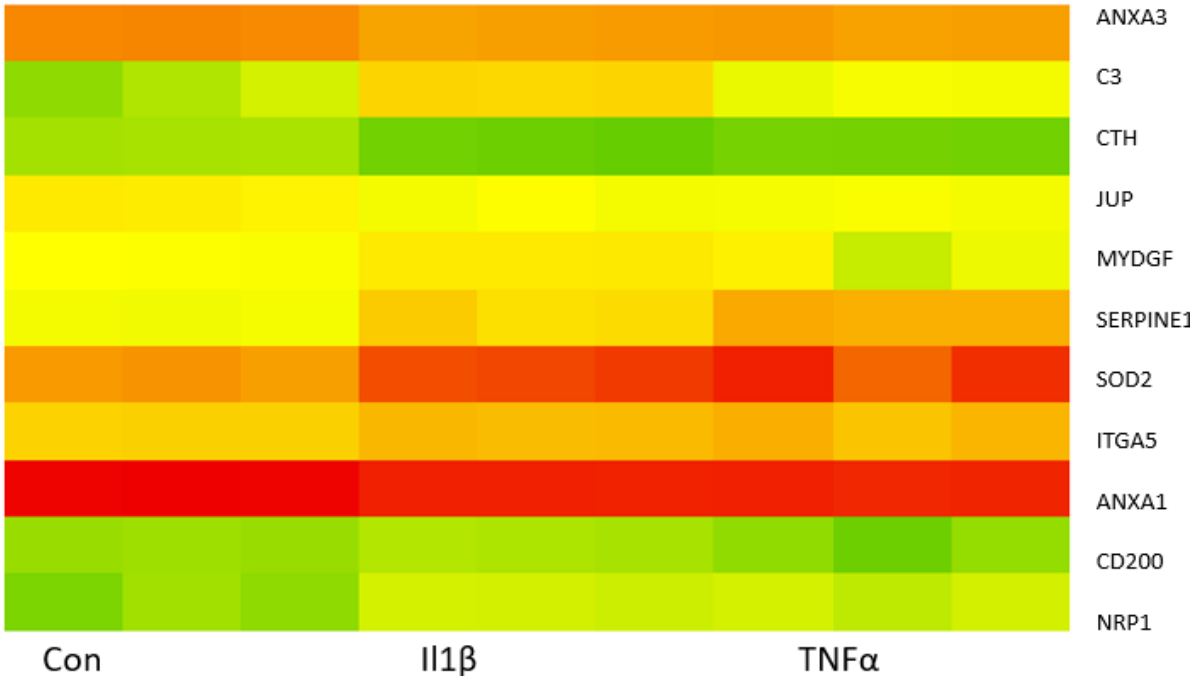


Figure 33 heatmap showing different expression level of angiogenic factors upon inflammatory stimulation

Angiogenic factors have higher LFQ Intensities upon stimulation with either Il1β or TNFα, compared to control group. Proteins involved in Epithelial to mesenchymal transition are found significantly regulated. **CRB2,** involved in EMT during development, is found upregulated , while **DAB2** and **SMAD 3** are found downregulated (Figure 34)

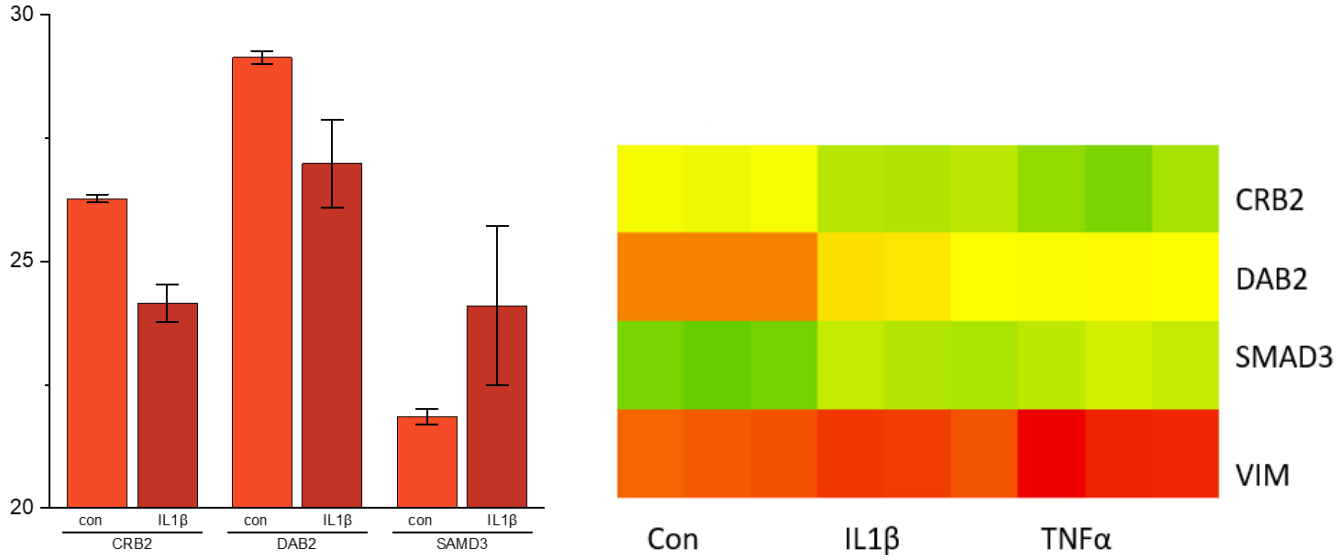


Figure 34 heatmap showing proteins involved in EMT

Stimulation with TNFα resulted in higher LFQ Intensities, in expressing classical EMT marker **vimentin.** While stimulation upon IL1β didn't result in higher LFQ intensities of VIM compared

to control. Also, DAB2 and SMAD3 are higher expressed , according to their higher LFQ Intensities upon TNFα than with IL1β. According to Cytoscape analysis, EMT was found significantly upregulated upon TNFα stimulation. For example, **tenascin** has been found upregulated upon stimulation of both IL1β and TNFα. Tenascin is a specific extracellular matrix protein implicated in guidance of migrating neurons as well as neuronal regeneration. It is a ligand for many integrins and stimulates angiogenesis by elongation, migration and sprouting of endothelial cells. (*125*) Proteins involved in extracellular matrix interactions and components, such as **laminins** , **collagens** and **integrins** have been found upregulated (data not shown) in the cytoplasmic fraction of the 12-Z cells upon respective inflammatory stimulation

6.1.4 Stimulation with TNFα

A total of 4250 proteins were identified, while 510 proteins were found regulated upon TNFα treatment. Inflammatory signature was visible as mentioned before. Cytoscape analysis showed a high number of various pathway cluster including Vascular endothelial growth factor receptor signaling pathway, metabolic pathways, angiogenesis and EMT and vestiulocholear nerve structural organization. Nervous interaction cluster contained various pathways involved in neuron recognition, integrin mediated signaling pathway, semaphorin signaling and VEGF signaling.

6.1.4.1 Neuron interactions:

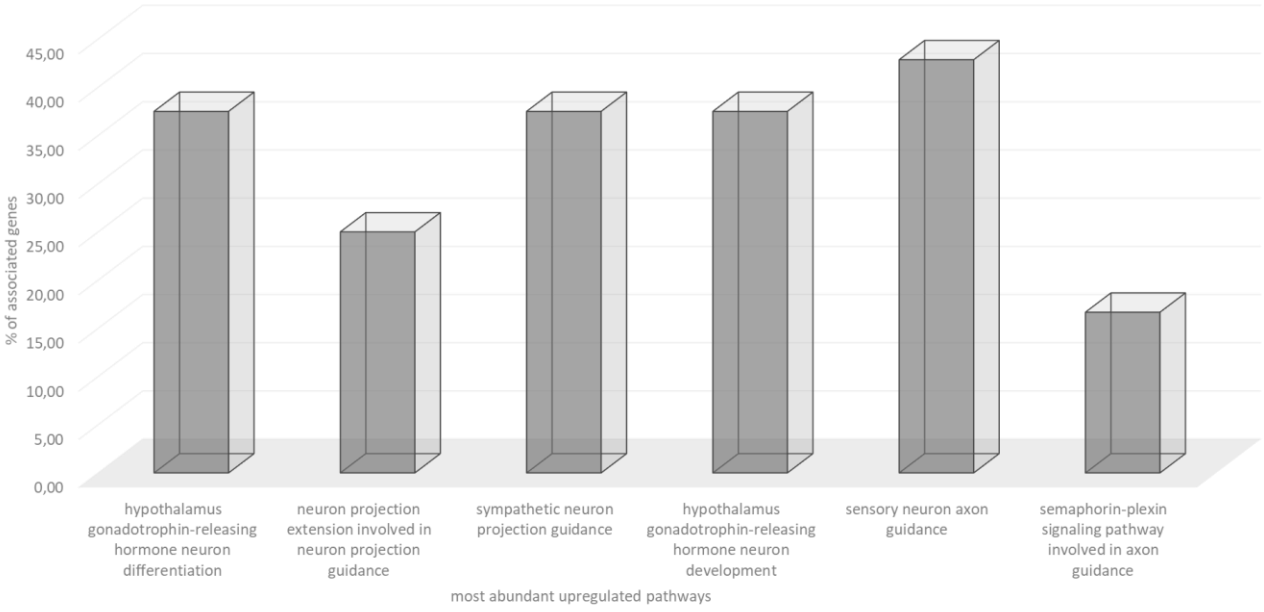


Figure 35 most abundant upregulated pathways in neuronal interactions

Figure 35 shows the most abundant upregulated pathways involved in neuronal interactions and their respective % of associated genes according to GO. Sensory neuron axon guidance displayed over 50% of associated genes, followed by sympathetic neuron projection guidance with over 40% of associated genes. Semaphorin-plexin signaling pathway, involved in the pathogenesis of endometriosis (79), has been found upregulated with 20% associated genes.

Prominent proteins involved in semaphorin signaling **NRP1** and **NRP2** are found upregulated upon stimulation with TNFα. Semaphorin signaling has been found abundantly upregulated in various signaling pathways such as semaphorin receptor activity, semaphoring-plexin signaling pathway involved in axon guidance and semaphorin-plexin signaling pathway involved in neuron projection guidance. Proteins found upregulated involved in semaphorin signaling contained **DPYSL3, RPL10, DPYSL2, EPHA4** among others. Figure 37 shows a heatmap displaying LFQ differences upon stimulation with TNFα compared to non-treated endometriotic control.

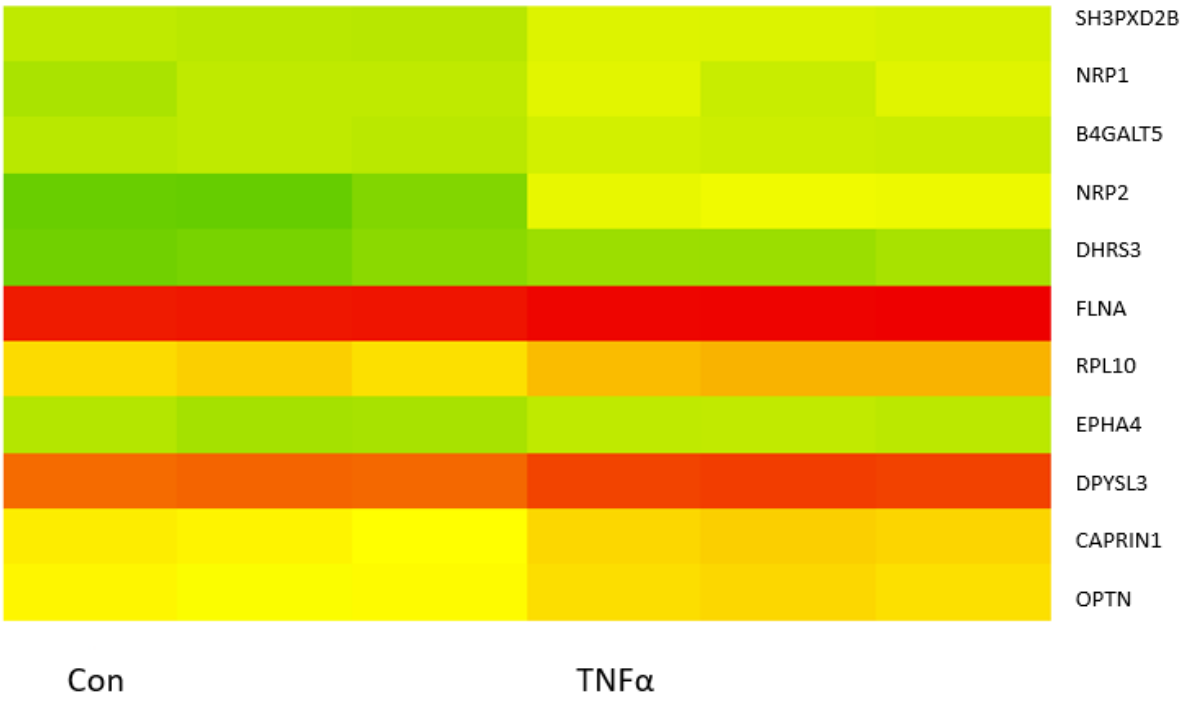


Figure 36 heatmap showing proteins involved in neuronal interactions upon stimulation of TNFA

As seen with Figure 36 proteins like **FLNA** and **DPYSL3** display already high LFQ Intensities in the non-targeted endometriotic control, upon inflammatory stimulation. A closer look on proteins involved in neuronal interactions, reveals similarities to embryonic neuronal development, as some of the proteins functions are involved in embryonic brain development.

6.1.4.2 Embryonic development

According to Cytoscape analysis pathways involved in embryonic development were found significantly upregulated. As shown in figure 37 different embryonic expression patterns are activated and found upregulated. 62% of all pathways found upregulated involved in embryonic development are involved in embryonic dorsal root ganglion signaling.

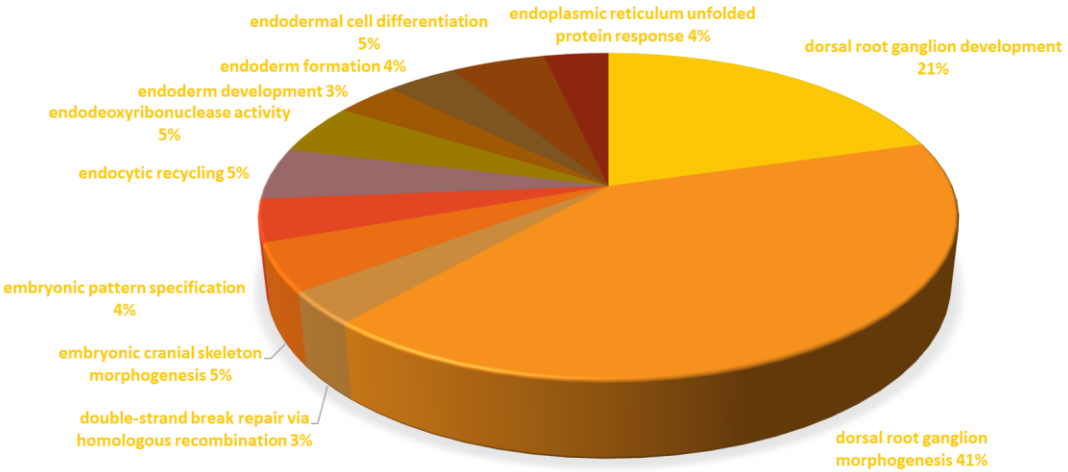
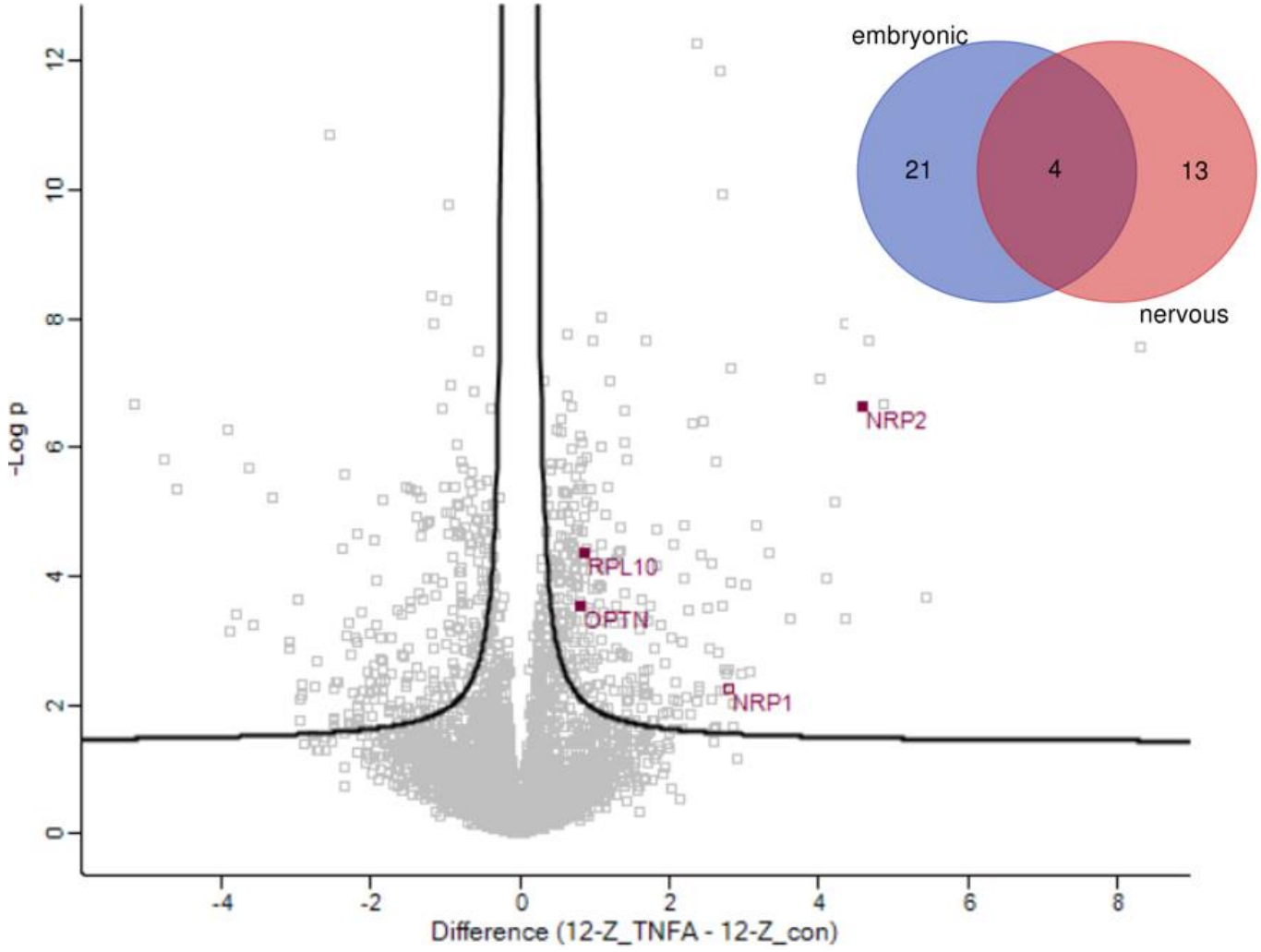


Figure 37 pathway analysis of embryonic development

41% of associated genes involved in dorsal root ganglion morphogenesis have been found significantly upregulated compared to non-treated endometriotic control. Dorsal root ganglion morphogenesis is a crucial step in neuronal development. It develops in the embryo from neuronal crest cells. Cell bodies of sensory neurons known as first-order neurons are embedded in the dorsal root ganglia. (*126, 127*) Some proteins involved in dorsal root ganglion morphogenesis are also involved in neuronal interactions pathway analysis (figure 38).

24% of proteins involved in nervous interactions are also involved in embryonic development pathway signaling. **NRP2** and **NRP1** involved in semaphorin signaling correlate with dorsal root ganglion morphogenesis as NRP1 and NRP2 are highly expressed in neuronal development. **OPTN** and **RPL10** play an important role in embryonic brain development. (*128*)

Figure 38 proteins involved in both embryonic development and nervous interactions; volcano plot showing involved proteins FDR=0.5;S0=0.1



6.1.4.3 Epithelial cell migration:

Proteins involved in embryonic development and neuronal developmental signaling are also involved in epithelial cell migration, according to Cytoscape analysis. **NRP1** and **NRP2** ,**RPL10** have already been implicated in semaphorin signaling. Figure 40 shows the significant upregulation of proteins involved in epithelial cell migration upon stimulation with TNFα.

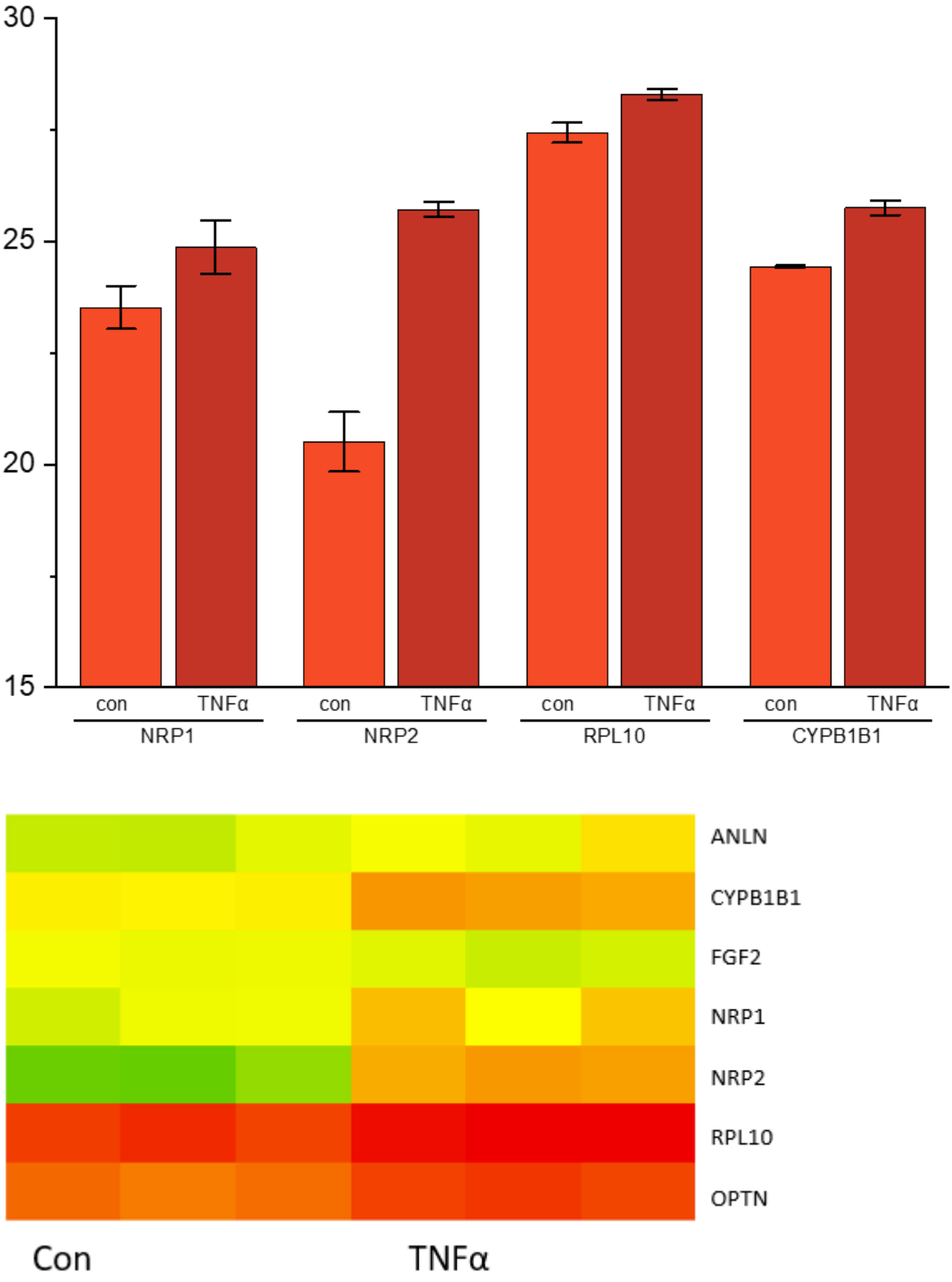


Figure 39 upregulated protein (log2 LFQ Values) ; heatmap showing higher LFQ levels upon stimulation

CYB1B1 , **ANLN**, **FGF2** and **OPTN** have also been found upregulated upon stimulation with tumor necrosis factor. RPL10 and OPTN display high LFQ intensities even without simulation, but still a significant upregulation upon treatment.

Many different genexpression patterns have been found upregulated upon stimulation with TNFα. Figure 40 shows an overview on the most abundantly regulated signaling pathways and their respective proteins.

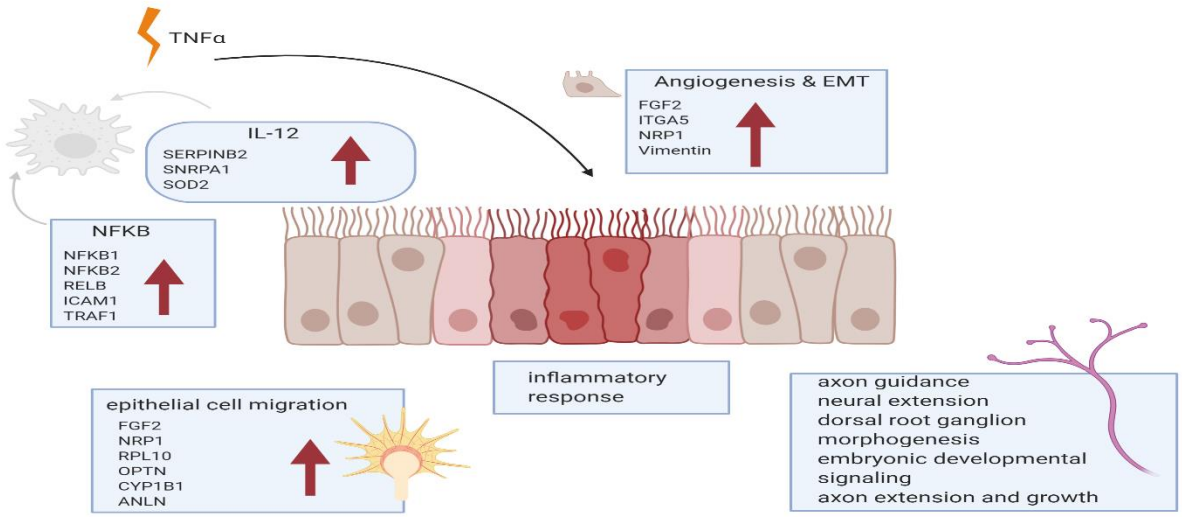


Figure 40 overview on stimulation with TNFα

Stimulation with TNFα induced genexpression of proteins involved in Angiogenesis and epithelial to mesenchymal transition. Expression of EMT marker **Vimentin** displayed a higher LFQ Intensity upon stimulation with TNFα than with IL1β. Acute inflammatory response was found regulated involving genexpression regulation of **ICAM1**, **VCAM1. SERPBINB1, NFKB1, NFKB2, RELB; CD14** and many more. Various interleukin signalings have been found regulated, whilst **IL-12** signaling was the most abundantly regulated. In General, stimulation with TNFα resulted in numerous neuronal signaling cascades and embryonic neuronal dorsal root ganglion development. Semaphorin signaling involving **NRP1, NRP2** and upregulation of **VEGF signaling pathway** (data not shown), was induced. Stimulation with TNFα resulted in more distinct developmental process regulation than IL1β. Epithelial cell migration upregulation correlates with both embryonic developmental and neuronal signaling.

6.2 Proteomic analysis of endometrial cell line THESC

Overall the proteomic profiles created upon different stimulations is reflected in the observed data. Proteomic analysis of the THESC cells both with TNFα and IL1β stimulation resulted in total amount of 2607 proteins A Principal component analysis (shown in figure 41) shows a separation of the samples upon their different respective inflammatory activation. Inflammatory stimulation with Il1β is shown in red , while TNFα stimulation is colored in blue. Both inflammatory activated batches are separated from control group shown in green.

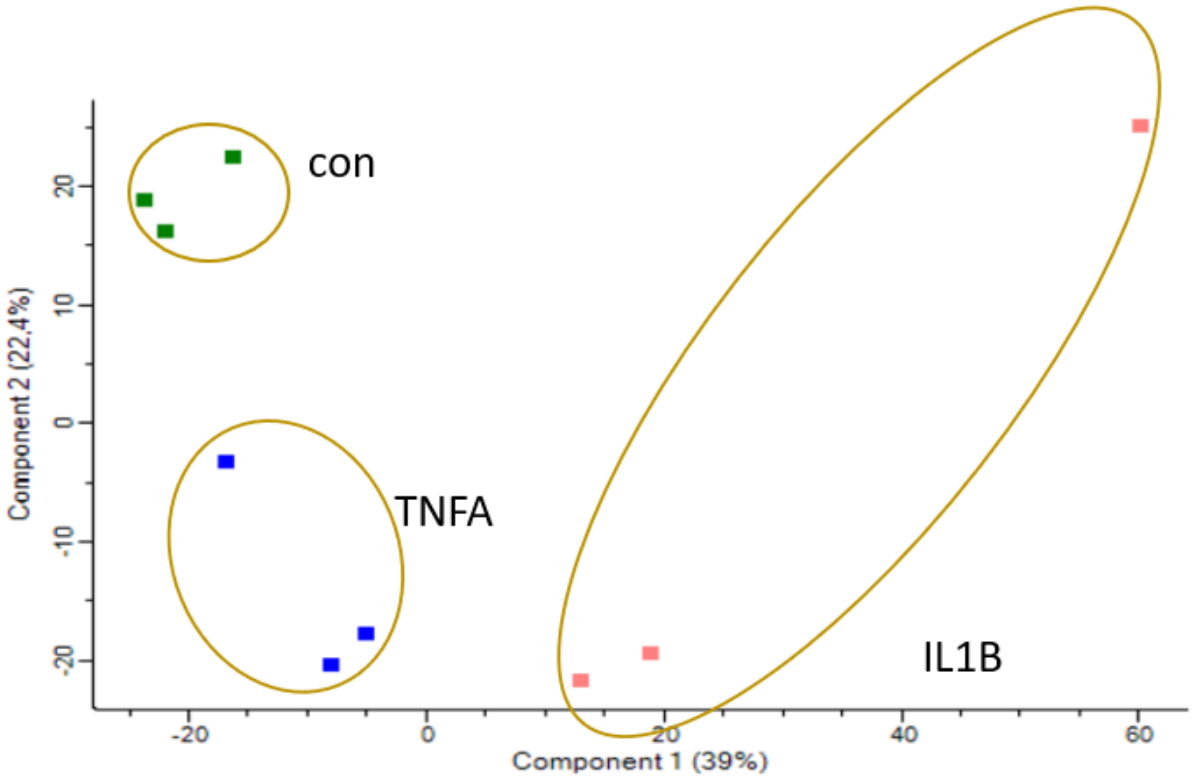


Figure 41 Principal component analysis of THESC

Stimulation with IL1β and TNFα resulted in a perfect separation compared to the control (non-treated) group, which is not inflamed, due to the fact that THESC cell line is a healthy endometrial fibroblast like cell line. Stimulation with TNFα resulted in less regulated proteins (510) than IL1β stimulation (544 regulated proteins). Generally, the analysis of inflammatory activated and endometrial fibroblast cells without treatment showed different alterations in the proteome profile, but mostly inflammatory behavior.

6.2.1 Stimulation with IL1β:

The proteomic analysis of the inflammatory activated endometrial fibroblast cells showed a reduction in proteins important for synthesis of cholesterol and steroids. Pathways within the biosynthetic cluster regulating sterol biosynthetic processes were found abundantly regulated. Stimulation with Il1β shows a similar direction compared to the 12-Z endometriotic epithelial cell line, but different than literature. Upon stimulation with Ilβ cholesterol biosynthesis is found downregulated. Estradiol signaling has been found downregulated.

ACTR1A , CALR, CCN2, SRC, MITCO2P12 are significantly downregulated as shown in figure 42.

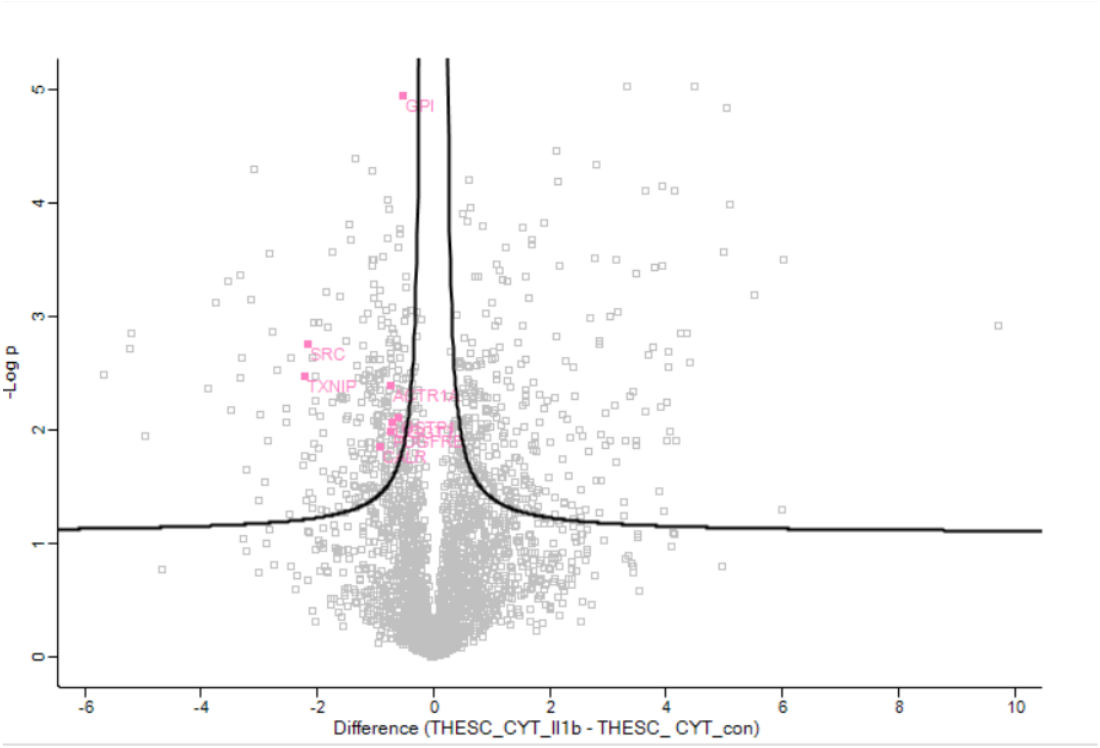


Figure 42 volcano plot showing proteins involved in estradiol signaling; FDR=0.5;S0=0.1

Response to corticosteroid and TGFβ pathways have also been found downregulated upon stimulation with IL1β.

In general, stimulation with an inflammatory agent resulted in downregulation of different pathways involved in pathogenesis of many diseases. Contrary to the 12-Z cell line sprouting Angiogenesis has been found downregulated as well as regulation of epithelial cell migration, response to estradiol and corticosteroids and neuronal interactions**. NRP2 , ITGB1, IQGAP1, APEX1** and **SOD1** have been found significantly downregulated compared to non-treated endometrial control.

According to Cytoscape analysis, 691 upregulated pathways, where found upon stimulation with IL1β. Most of them involved in metabolic, DNA replication and ribosomal processes. Most abundantly regulated pathways are shown in figure 44. Stimulation with an inflammatory agent resulted in downregulation of nervous system processes and VEGF receptor signaling pathway, contrary to the 12-Z findings. Epithelial cell migration has been found downregulated both sides with upregulated and downregulated proteins. Ovarian follicle development as well as inflammatory response reactions including ERK cascades and natural killer cell activation have been found upregulated. These upregulations have only been found upon stimulation of IL1β in THESC and never with 12-Z.

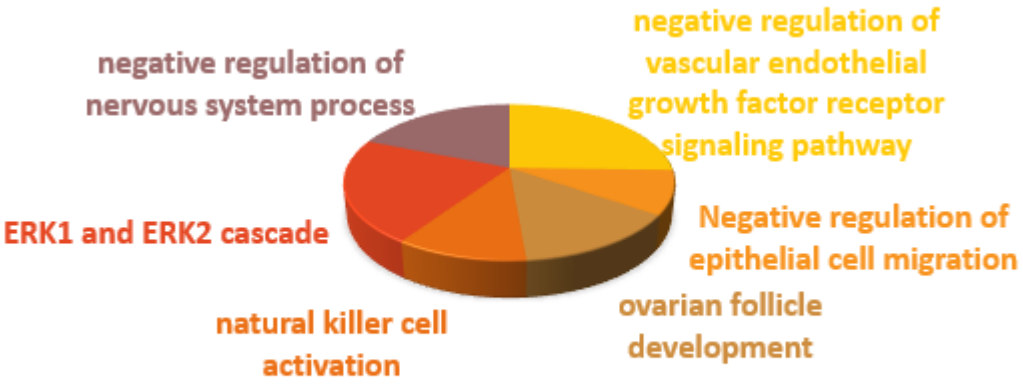


Figure 43 upregulated pathways (GO)

6.2.2 Stimulation with TNFα:

In General, stimulation with TNFα resulted in 510 regulated proteins, but 50 upregulated pathways identified. Most of the pathways identified were involved in metabolic processes and DNA replication. Most abundantly identified pathways were hyaluronan catabolic process and response to IL-12 as well as inflammatory signature pathways. Cholesterol biosynthesis has not been found, neither neuronal interactions. **HLA-B ;NFKB1 , RALA, SERRPINB2** and **SOD2** have been found upregulated. **CD9 , PDGF** and **CD81** have been found downregulated , resulting in negatively regulated extracellular vesicle transport. In contrary in 12-Z both IL1β and TNFα resulted in upregulation of CD9 and CD81.

6.2.3 Summary of proteomic results:

In General, THESC and 12-Z cells responded differently towards inflammatory stimulation. An overview on the different responses is shown in Figure 44. Endometriosis cell line upregulated neuronal signaling and embryonic developmental genexpression, upon TNFα as well as angiogenesis and EMT. Stimulation with IL1β on endometriosis epithelial cells resulted in upregulated wound healing signaling pathway, angiogenesis and EMT while cholesterol biosynthetic process was found significantly decreased.

On the other hand, healthy endometrial cell line THESC responded notedly differently. IL1β resulted in downregulation of nervous interactions , angiogenesis and upregulation of proteins, which negatively regulate angiogenesis , nervous system processes and mitotic cell division as well as proliferation. Natural killer cell activation, NFKB , IL-12 signaling have been found upregulated , upon inflammatory stimulation, hence indicating inflammatory response. Stimulation with TNFα resulted in few regulated pathways. Inflammatory response has been found upregulated as well as hyaluronan metabolic process. Findings tend to differ from stimulation of the 12-Z cells.

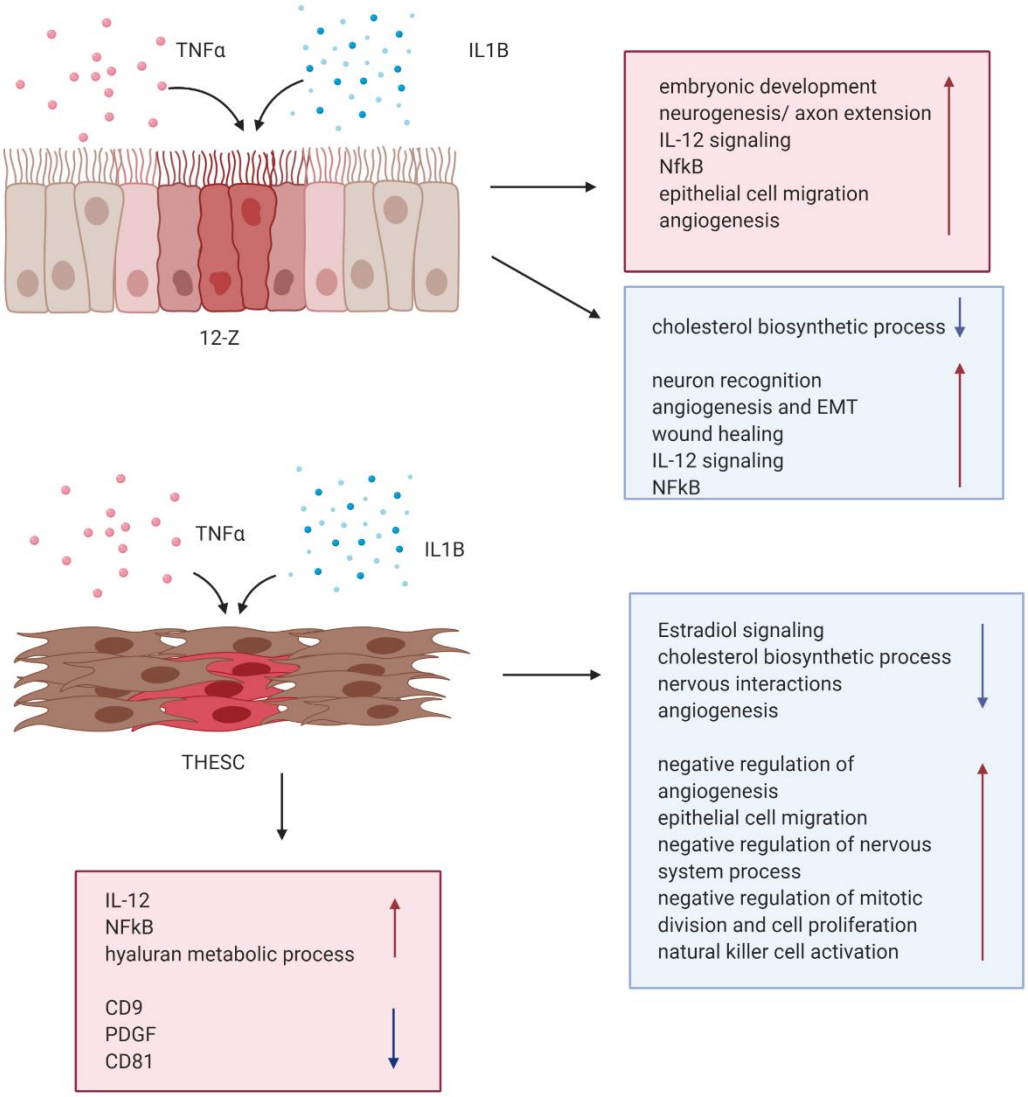


Figure 44 differences between cell lines

7. Discussion and Conclusion:

Endometriosis is a chronic inflammatory disease with a still unknown pathogenesis. Angiogenesis, EMT and neurogenesis are one of the main pathologic features endometriotic cells are capable of. (2) This master thesis showed assessment of pathogenesis and inflammatory response using a model of endometriotic epithelial cell line 12-Z and a healthy control cell line THESC. Response towards IL1β and TNFα secreted by macrophages in vivo, revealed upregulation of angiogenesis and EMT upon respective inflammatory stimulation. In contrary to healthy endometrial cell line THESC. Although these in vitro experiments are not able to completely cover the complexity of the in vivo situation to its full extend it provides valuable insight into the ongoing molecular mechanisms occurring during inflammatory stimulation. Therefore, this thesis provides the foundation for further investigations with primary patient materials.

7.1 Estrogen and cholesterol biosynthesis:

In situ estrogen synthesis and metabolism is one of the most important features endometriotic cells exhibit, since the disease is estrogen dependent. Estrogen as well as other steroid hormones are synthesized from cholesterol. Hence cholesterol biosynthesis is a crucial factor in endometriosis development. As previously described the aromatase pathway is crucial for endometriosis estrogen metabolism. Neither StAR nor CYP19A1 (aromatase) have been found analyzing the endometriotic cell line 12-Z. Since CYPs are hard to detect using mass spectrometry due to the fact that they are mostly membrane bound and display low abundance it is not sure to determine whether such enzymes were present or not.

In postmenopausal woman estrogen is produced by adipose tissue , where formation of estrogen is regulated by cytokines such as IL1β , TNFα and IL-6. (*129*) Upon stimulation with IL1β cholesterol is being transferred from the cytoplasm to mitochondrion for steroid biosynthesis. (40) However, as data suggest, cholesterol biosynthetic enzymes are being downregulated upon stimulation with IL1β. **AKR1B1, CYP51A1, DHCR24, FDFT1, HMGCS1, IDI1, MSMO1, SCP2, SQLE, FASN** and **ACACA** are being downregulated in endometriotic epithelial 12-Z cells. AKR1B1 for example, is a necessary enzyme for reduction of steroids and their derivatives and prostaglandins. (*130*) Stimulation with IL1β led to downregulation compared to non-treated endometriotic control.

Since, endometriotic lesions have their own estrogen metabolism independently from the menstrual cycle, IL-1β might not be the main trigger for cholesterol biosynthesis here. Hence, cell culture conditions only reflect the in vivo situation to a certain degree. Since, in vivo the endometriotic lesions are supplied with DHEA produced by stromal fibroblasts, PGE2 coming from macrophages and many more cytokines. (*79*) Thus, for a better reflection in the estrogen metabolism of endometriotic cells, further investigation is needed. Precise co-stimulation and specific enrichment strategies for CYP19A1 and StAR collection are needed.

Interestingly, biosynthesis as well as estradiol synthesis was downregulated in human endometrial cell line THESC as well. This might be due to downregulation of estradiol and

upregulation of progesterone for establishment of an upcoming pregnancy. IL1β is one of the main cytokines produced at the uterine-embryonic interface during implantation. The presence of IL1β is involved in the immunotolerance at the maternal-placental interface and is needed for establishing a pregnancy through modulation of the nuclear factor kappa-B (NFκB) system, for regulation of the progesterone receptor expression and NFKB activation for endometrial receptivity. (*131*)

Correlatingwith the data,NFκB signaling pathway was found upregulated indicating uterine endometrial cells were preparing for pregnancy by downregulation of estradiol signaling and attraction of immune cells such as natural killer cells. Natural killer cells are present in the human endometrium prior to the initiation of pregnancy and are attracted at high levels more granulated during the progesterone dominated secretory phase after ovulation and throughout the first trimester. Uterine natural killer cells are nothing like other tissue resident natural killer cells, they have a specific phenotype residing in the decidua. They predominantly produce cytokines and display low cytotoxicity. They mostly provide the uterine microenvironment with cytokines and chemokines attracting macrophages mediating a key role for a successful pregnancy. (*132, 133*) One major reason for endometriosis related infertility is implantation failure due to aberrant function of uterine natural killer cells . The mostly lose their "friendly" phenotype and exhibit higher cytotoxicity even against the fetus. (*134*) Correlating with the Data natural killer cell activation has only been found upregulated with healthy endometrium cell line THESC, never with 12-Z endometriotic cells.

7.2 Angiogenesis and EMT

Furthermore, another pathological event happening in endometriosis is the development of new blood vessels from pre-existing ones, called angiogenesis. Initiated by angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and release of matrix metalloproteases (MMPs) for degradation of the vessel basement membrane. Endothelial cells migrate into the surrounding tissue, resulting in the formation of sprouts. Sprouting angiogenesis is the most important mechanism for the formation of new blood vessels and is an efficient strategy of growing tumors.

In endometriosis the regulation of angiogenesis shares many similarities to the mechanism mediating the pathological angiogenesis of tumors and metastases. Despite secretion of VEGF, promotion of angiogenesis in endometriosis include factors like FGF. (*135*)Corresponding to data **FGF2** had been found upregulated in both TNFα and IL1β stimulation, since sprouting angiogenesis signaling had been found significantly upregulated in the 12-Z endometriotic cells upon stimulation with inflammatory agents.

Although VEGF wasn't detected directly, due to the fact that it would probably be in the secretome fraction of the cells and not in the cytoplasmic fraction as investigated, VEGF dependent signaling pathway was found significantly upregulated upon stimulation with TNFα. **ICAM1, VCAM-1,** important factors for cell-cell interactions happening in vasculogenesis, are found upregulated as well upon stimulation with TNFα. In vivo estrogen

signaling plays a major role in endometriosis angiogenesis , since it induces the genexpression of more angiogenic factors and MMPs. MMPs could not be detected using mass spectrometry based proteomic analysis of the cytoplasmic fraction, since they would secreted.

However, **Tenascin** marker for angiogenesis and EMT normally expressed by myofibroblasts (*136*)was found upregulated as well as **Laminins**, **Integrins** and **collagens** important for extracellular matrix reorganization in angiogenesis. (*135*) Angiogenesis is a crucial step in endometriosis, as it is necessary to establish the disease. According to Sampson theory of retrograde menstruation, menstrual fluid containing endometrial cells are being transported via the fallopian tube into the pelvic area (15). There, endometriotic cells epithelial and fibroblastic cells attach to other organs, needing various integrins. Expression of ICAM-1 is a highly important mechanism for adhesion to ectopic sides, also ICAM-1 expression inhibits immunosurveillance of natural killer cells, by hindering leukocyte-cell communication.(2,42)

In vivo macrophage attracting is the next step to fully undergo a constant proangiogenic genexpression, as they deliver more PGE2 and VEGF as well as TNF-α, thus resulting in a constant stimulation of angiogenesis (2,41,44) Data, collected within this thesis, suggests higher angiogenic potential upon stimulation with TNF-α. **ICAM-1** expression as well as **VCAM1** expression was significantly found more regulated with TNFα than with IL1β stimulation. This is mostly regulated by NFκB signaling pathway activation.

In endometriosis TNFα induced IKK dependent NFκB pathway is mostly activated (49,50). Since NFκB is cyclically active during menstruation it is not surprising that non-endometriosis cell line THESC exhibited high induction of **NFκB** activation upon inflammatory stimulation with both IL1β and TNFα. It has been known for long time that during the normal human menstrual cycle changes in cytokine concentration are happening (*137*). Changes are commonly noticeable in the luteal phase of the ovulatory menstrual cycle, including rise of body temperature , leukocyte migration from vascular compartments to the endometrium. Therefore, secretion of Interleukins and TNFα takes place (*138*).

In endometriosis however, NFκB stimulation takes place constantly within endometriotic lesions (53). **NFκB2** and **RELB** as well as **NFκB1** were found statistically upregulated. Therefore, it is interesting that in the endometriosis cell line 12-Z NFκB activation took place by activating non-conical NFκB pathway and canonical NFκB pathway In the THESC cells however, non-canonical NFκB pathway was not activated.

Furthermore, another pathologic feature endometriosis cells are using for survival during anoikis, which is the implantation at inappropriate locations, is epithelial to mesenchymal transition. EMT is a process in which epithelial cells lose polarity and cell-to-cell contacts in order to acquire migratory invasive abilities of mesenchymal cells. Endometriotic cells need these abilities for establishment of lesions*139*). (It is not yet known what distinct EMT signaling pathways are activated in endometriotic lesions, but it is clear that EMT is essential for the development of endometriosis, since cells would die when they detach from the extracellular matrix. One possible explanation in EMT might be due to the fact that during the

embryonic development of the urogenital system, endometrium is derived from intermediate mesoderm via mesenchymal to epithelial transition (MET). Endometrial epithelial cells might have a tendency to return to their original state via EMT (*140*).

Data suggest that upon stimulation with TNFα and IL1β EMT is induced in endometriotic cells in contrary to non-endometriosis cell line THESC, where EMT was not found significantly upregulated. Upregulation of **SMAD3** and **CRB2** in both TNFα and IL1β stimulation, might indicate a potential tendency toward rest activity of embryonic signaling. Since, CRB2 plays a central role during the epithelial-to-mesenchymal transition at gastrulation and promotes ingression, which is the process of cells leaving the epithelial epiblast and move inside the embryo to form a new tissue layer*141*). (SMAD3 is also a key promoter in EMT, especially after injury (*142*). Upon stimulation with TNFα epithelial to mesenchymal transition marker **VIM** is highly expressed in endometriosis cells, while Il1β stimulation does not result in it. Thus, EMT could be especially induced by TNFα, since in vivo TNFα signaling is one of the major communication loops macrophages and endometriotic lesions have. As an Li et al. showed , macrophages equally induce EMT in endometriosis and especially adenomyosis (endometriosis within the uterine muscle wall) by finding SMAD3 and VIMENTIN being upregulated. This correlates with the data presented, as TNFα stimulation should mimic macrophage stimulation happening in vivo (*143, 4, 144*).

However, findings differ for the healthy endometrial cell line THESC , since angiogenesis is not upregulated, but is found statistically significantly downregulated upon stimulation with IL1β. Thus suggesting that angiogenesis needed for implantation is mostly induced by the embryo secreting VEGF and other angiogenic factors (*145*) . These findings might suggest a possible tendency of endometriosis cells towards embryonic genexpression during EMT and angiogenesis, while healthy endometrial cells might try to avoid distress for establishment of

an upcoming pregnancy. A possible mechanism for Invasion and progression of endometriosis is shown in figure 45.

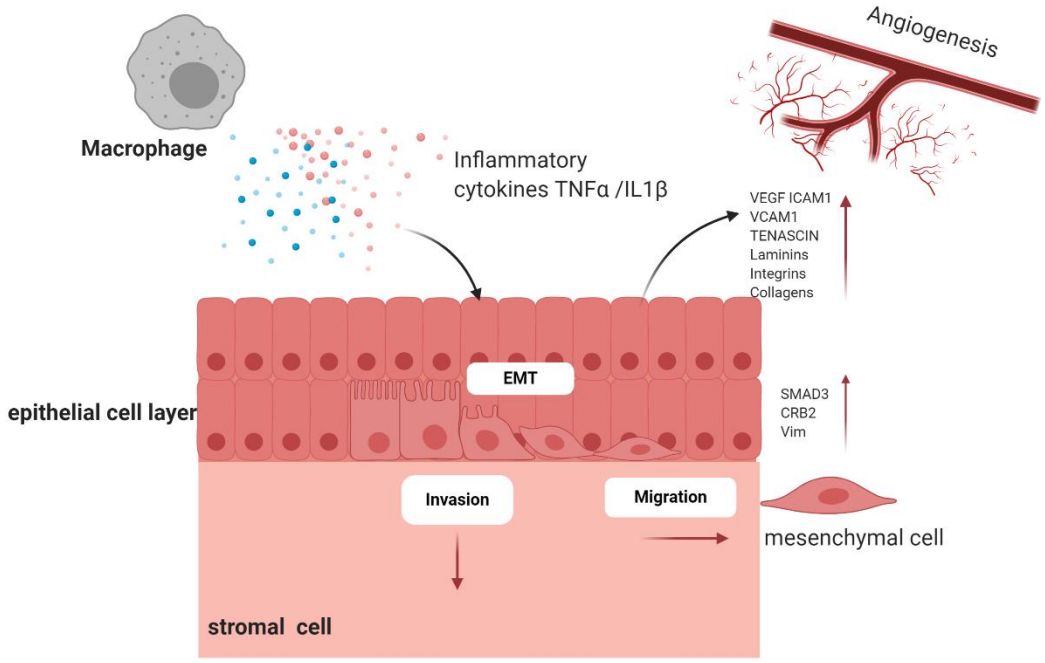


Figure 45 possible mechanismn in vivo

7.3 Neurogenesis

Neurogenesis is the process of neuron generation , coming from neural stem cells. It is most active during embryonic development and is responsible for production of all different types of neurons of the organism. After birth, neurons stop mitosis and live on the entire lifespan of the human (*146*). After a neuron is created it migrates long distances to their final destination maturing and generating axons and dendrites. Adult neurogenesis is a rare event only happening in neuroepithelial cells passing through neurogenic divisions. The adult subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus of the hippocampus are the only two regions, adult neurogenesis is happening (*147–149*).

Once neurons have developed in embryonic development, their axons need to grow reaching the correct targets. This target finding process is called axon guidance, happening in neural development. Signals can repel or attract axons. There are different signaling cascades and receptors important for axon guidance, netrins, slits , cell adhesion molecules (CAM) ephrins and semaphorins. Semaphorin signaling activates cell surface receptors called Plexins and neuropilins. In addition to the main axonal guidance signaling, developmental growth factors influence axonal growth, such as NGF, laminins , tenascin and FGF (*150*). During embryogenesis growth of nerves and blood vessels is a combined event, called neuroangiogenesis. In endometriosis neuroangiogenesis is a major pathologic event(*151–153*). Neuropilins and VEGF act as neurotrophic and neuroprotective mediators. Their synergistic effect enhances migration and proliferation of vascular development and axon guidance. (*151*)

Corresponding to the data presented **Laminins, Tenascin** and **FGF2** were found upregulated in endometriotic 12-Z cells upon stimulation with IL1β and TNFα. Suggesting that axonal guidance would possibly happen. According to Cytoscape analysis various pathways and signaling cascades concerning neurogenesis and axon guidance were highly upregulated. Proteins involved like **NRP1** and **NRP2** as well as **DPYSL3** play a major role in semaphorin signaling(*154*). This suggests that semaphorin signaling is active upon stimulation with TNFα in the 12-Z endometriotic cells. It is not known how well an in vitro culture model portrays an in vivo situation of combined events such as macrophage stimulation and nervous signaling. But still neuroangiogenesis was visible upon stimulation with TNFα, since both Angiogenesis, **VEGF signaling pathway** and neurogenesis including **axonal guidance**, by **semaphorin-plexin signaling** was found upregulated.

In vivo it has been postulated that macrophage stimulation upon endometriotic cells, lead to secretion of neuroangiogenic factors and more estradiol, thus enhancing secretion of neuroangiogenic factors of macrophages. (*155*) As described previously macrophage neuron crosstalk leads to an aberrant innervation into endometriotic lesions, due to estradiol signaling. Estrogen acts on nerve fibers resulting in release of different molecules promoting the recruitment of macrophages, which will then lead to infiltration of macrophages into peripheral nerves. Macrophage signaling upon estradiol stimulation results in neuroangiogenesis and promotes neuronal survival and sympathetic axonal growth into the endometriotic lesions. Semaphorin signaling coming from macrophages is known to be involved in endometriosis by NRP1 signaling between nerve cells and endometriotic macrophages.(77-79)

Therefore, the most common model for neuroangiogenesis has been by macrophage attraction. Since 12-Z endometriotic cells produce high level of Neuropilin receptors, it may be that macrophage mediated attraction of nerve cells is because of endometriotic epithelial cell mediation. As the data presented clearly shows, 12-Z epithelial endometriotic cells are perfectly capable of axonal guidance and neuron projection extension involved in neuron projection guidance. **Semaphorin signaling** is found upregulated as well as various proteins involved in embryonic neuronal development. Such as **OPTN**, **EPHA4, DHRS3** among others. **Sensory axon guidance** was upregulated with over 50% of total gene association. Thus, macrophage interaction clearly is not the only responsible signaling event in endometriosis neurogenesis. Endometriotic epithelial cells attract nerve cells on their own. It might even be that macrophages are reprogrammed by the endometriotic lesion in order to help them attract nerve cells by supplying them with TNFα and macrophage derived neurogenic signaling molecules. It seems like endometriotic cells might not play an essential role in macrophage attraction of nerve cells, but are the main reason of it. Endometriotic epithelial cells clearly need stimulation of TNFα for axonal guidance and neuroangiogenesis happening, as data shows. Therefore, it might be that endometriotic cells sustain themselves by autocrine stimulation of TNFα in vivo , until they have attracted macrophages and reprogrammed them.

Endometriotic lesions might attract macrophages, reprogram them as the lesion wants them to be, completely changing macrophage function and enslaves them in order to sustain them with growth factors. This might be done upon semaphorin signaling. As data shows, **Semaphorin/NRP-1** signaling is found upregulated upon TNFα signaling. As already mentioned this signaling is involved in macrophage reprogramming (83). Macrophage reprogramming is an essential step for establishment of endometriotic lesions, since they provide lesions with growth and neuroangiogenic factors and help reprogramming other leukocytes preventing cytotoxicity towards endometriotic cells.(80) As shown in figure 10, macrophages change their polarity and behavior upon Semaphorin/Nrp1 signaling activating VEGF signaling pathway. Macrophage secretion of TNFα induces the sensory nerves to a constant induction of the action potential, by opening of sodium channels and leading to a vicious circle of TNFα secretion in endometriotic lesions, since these sodium channels can be activated upon TNFα secretion. Norepinephrine coming from nerve fibers can bind to estradiol-β2 receptor on macrophages, leading to activation of PKA signaling and thus stimulating TNFα mediated inflammation in endometriotic lesions. Therefore, inflammation is constantly starting all over again(80-85).

Therefore, this could possibly mean that TNFα stimulation coming from macrophages and via autocrine stimulation, is used for induction of macrophage reprogramming. Once macrophages are reprogrammed , they help endometriotic cells attract nerve cells and work synergistically in order for neuroangiogenesis to happen. Further investigations need to be done to confirm this theory, by co-culture experiments and primary cell culture and proteomic endometriotic tissue analysis. Non-endometriosis cell line THESC didn't exhibit upregulation of NRP1 nor any other form of neuronal growth signaling. Furthermore, neuron interactions were rather found significantly downregulated. Even more, 12-Z endometriotic cell line didn't feature upregulation of semaphorin signaling upon stimulation with Il1β. Suggesting that really TNFα stimulation leads to a possible induction of neuroangiogenesis, in endometriosis.

As data confirmed, 12-Z endometriotic cells displayed a high number of statistically significant upregulated proteins involved in neuron and axonal guidance, with over 50% of associated gens according to GO based Cytoscape analysis. Therefore, it might be possible, that endometriotic epithelial cells, feature the ability of attracting nerve cells and guide axons towards their lesions, on their own. They might not only supply macrophages with factors and estradiol, in order for them to perform neuroangiogenic signaling, but rather start neuroangiogenesis on their own, and even more enslave macrophages to supply them with TNFα in order to sustain neuroangiogenesis signaling. Macrophages might not be the main reason of neuroangiogenesis in endometriosis, but may rather function as an accomplice in crime. Figure 46 shows this possible hypothesis of how macrophages and endometriotic lesions work synergistically attracting nerve cells and leading to neuroangiogenesis.

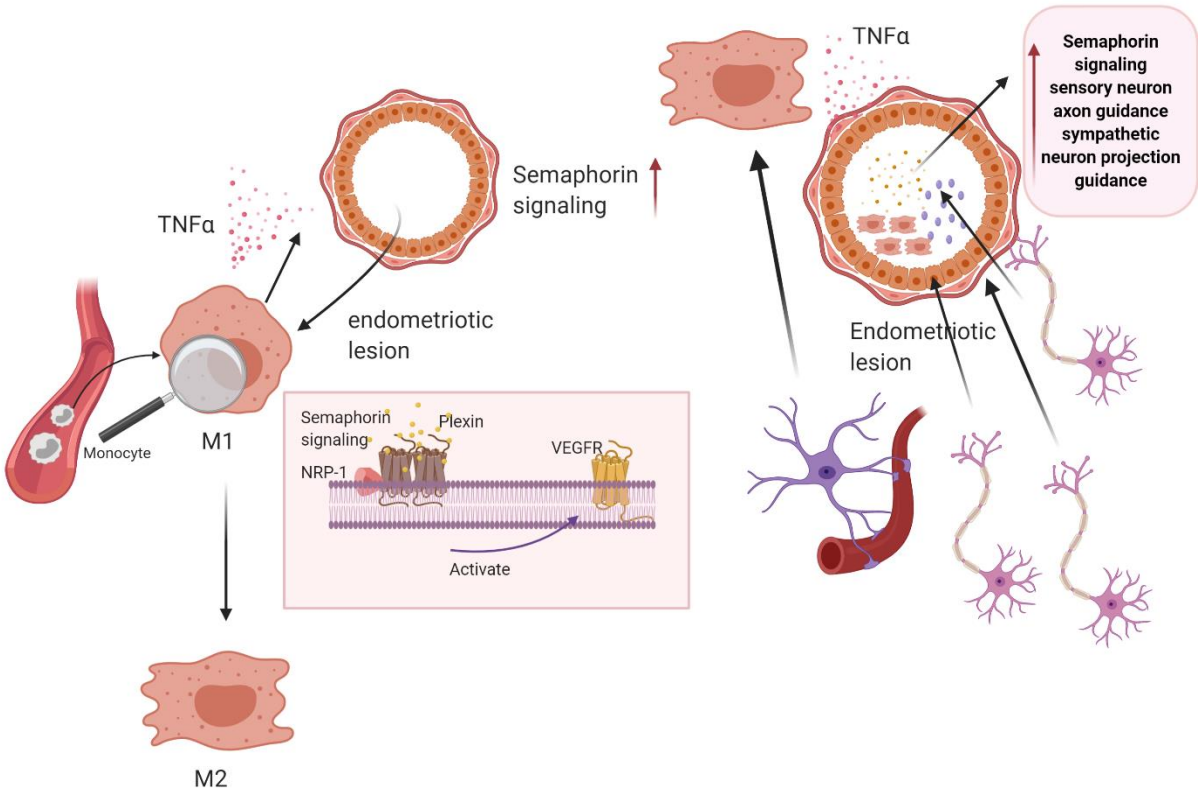


Figure 46 possible hypothesis of synergistic effect in neurogenesis between macrophages and endometriotic cells

Furthermore, data confirmed that upon stimulation with TNFα signaling pathways including sensory axon guidance and sympathetic neuron projection were significantly found upregulated. Interestingly, proteins involved, were also found in embryonic developmental signaling. **OPTN, RPL10** and both **NRP1** and **NRP2** are involved in embryonic brain development. (*156*) Stimulation with TNFα resulted in a highly abundant embryonic expression pattern, with various pathways upregulated. The most abundant upregulated pathway however, is involved in formation of the **dorsal root ganglion**. The dorsal root ganglion is a cluster of neurons (a ganglion) in a dorsal root of a spinal nerve. Sensory neurons a most located in the soral root ganglia. It develops in the embryo from neural crest cells. (*157*).

Stimulation with IL1β led to an activation of embryonic development as well, but TNFα however resulted in more distinct neuronal developmental activation signaling. **Integrins** involved in axon guidance were found upregulated like **ITAG5** also involved in angiogenesis (*158*). Signaling pathways upregulated upon stimulation of TNFα resulted in activation of **embryonic developmental signaling**. However, it seems that uptake of TNFα induces activation of embryonic genexpression signaling involved in neuronal development. Since, neurogenesis is a process mostly observed in embryonic development, it makes sense that embryonic developmental signaling might be activated. This data shows at least that the 12-Z epithelial cell still resemble at a high degree the primary endometriosis epithelial cells collected.

Since, THESC didn't exhibit this kind of embryonic signaling activation. Embryonic signaling was found upregulated according to the biological function of endometrial stromal cells supporting and harboring a pregnancy , upon stimulation with IL1β. Pathways found upregulated involved in embryonic development were involved in **embryonic placenta morphogenesis**, involving proteins like **ETF1** and **IGF2**. Stimulation with TNFα didn't result in pathway upregulation involved in embryonic signaling, at all. All nervous interactions were found to be downregulated upon stimulation with IL1β in healthy endometrial cell line THESC, contrary to endometriosis cell line 12-Z. Embryonic neuronal developmental signaling activation might be the cause, of neuroangiogenesis of endometriotic epithelial cells.

Furthermore, Epithelial cell migration has been found upregulated upon stimulation with TNFα , in the data presented involving proteins such as **NRP1** , **NRP2** and **RPL10** , all involved in embryonic brain development as mentioned before (172) , as well as upregulation of **CYPB1B1**, which is involved in cell proliferation and metastasis through induction of EMT (*159*).

Since, neuroangiogenesis is a conserved embryonic developmental process (*151*) it is not astonishing, that embryonic expression pattern involved in neuronal signaling are being upregulated. There are different theories on how endometriosis develops, however there is evidence that a stem cell mechanism of tissue regeneration may exist in the cyclic endometrium (*160*) supporting the idea of stem cell derived endometriosis. Since both the outer layer functionalis and the inner layer basalis consist of primarily stromal and epithelial glandular cells , with embryonic Mullerian origin, it makes sense, that some sort of regeneration mechanism must take place after shedding during menses. (*161*)

However, as our data clearly states, it might somehow be that upon macrophage stimulation of TNFα endometriosis cells activate a dormant embryonic expression pattern, resulting in embryonic derived neuronal development. It might even be that small endometriotic lesions, might behave as an early stage embryo to a certain degree, since neutral killer cells express low cytotoxicity towards them and endometriotic lesions undergo processes important for embryonic development such as neuroangiogenesis and epithelial cell migration. Epithelial cell migration is a crucial and very complex step in gastrulation, in early embryonic life time. (*162*). Since many proteins were found upregulated coming from embryonic origin involved in other pathologic features of endometriotic cells, such as EMT , epithelial cell migration and neuroangiogenesis, it might be that somehow endometriotic cells experience a re-activation of embryonic genexpression , at least to a certain degree. It might be activated by some sort of stimulus, since compared to IL1β treatment, only TNFα stimulation achieved this high statistically abundant upregulation of neurogenesis and embryonic neuronal developmental signaling.

Endometriotic lesions might not only behave as an embryo it might be very reasonable, that the cells have adapted some of the embryonic signaling and reactivated it, since they are uterine cells, naturally interacting with an embryo. Since the only reason of endometrial cells

existing is reproduction and harboring a progeny it is possible that endometriosis has adapted embryonic behavior for survival. Endometriotic cells wander via the fallopian tube in order to implant themselves , in ectopic sides. A pregnancy is capable of implantation even outside of the uterus as ectopic pregnancies show. Therefore, it makes sense that the immunesystem doesn't recognize endometriosis as a threat but rather sustains it with growth factors angiogenic and neurogenic factors and feeds it with cytokines. Embryonic expression patterns are clearly found upregulated in endometriotic cells, as data confirms. Therefore, a possible theory of endometriosis might be that endometriotic epithelial cells re-activate upon whatever reason , embryonic gene expression at least to a certain degree , behave like a ectopic pregnancy when they implant outside of the uterus, attract macrophages and natural killer cells like the embryo would do. They reprogram macrophages in order to always be sustained with TNFα, as a main stimulus of neuroangiogenesis and other cytokines and growth factors. Endometriosis does not behave like cancer, since it is never deadly. Endometriosis behaves like a ectopic pregnancy struggling to stay alive , needing inflammation to implant attracting nervous cells of the patient. Figure 47 shows a possible theory how endometriosis might develop.

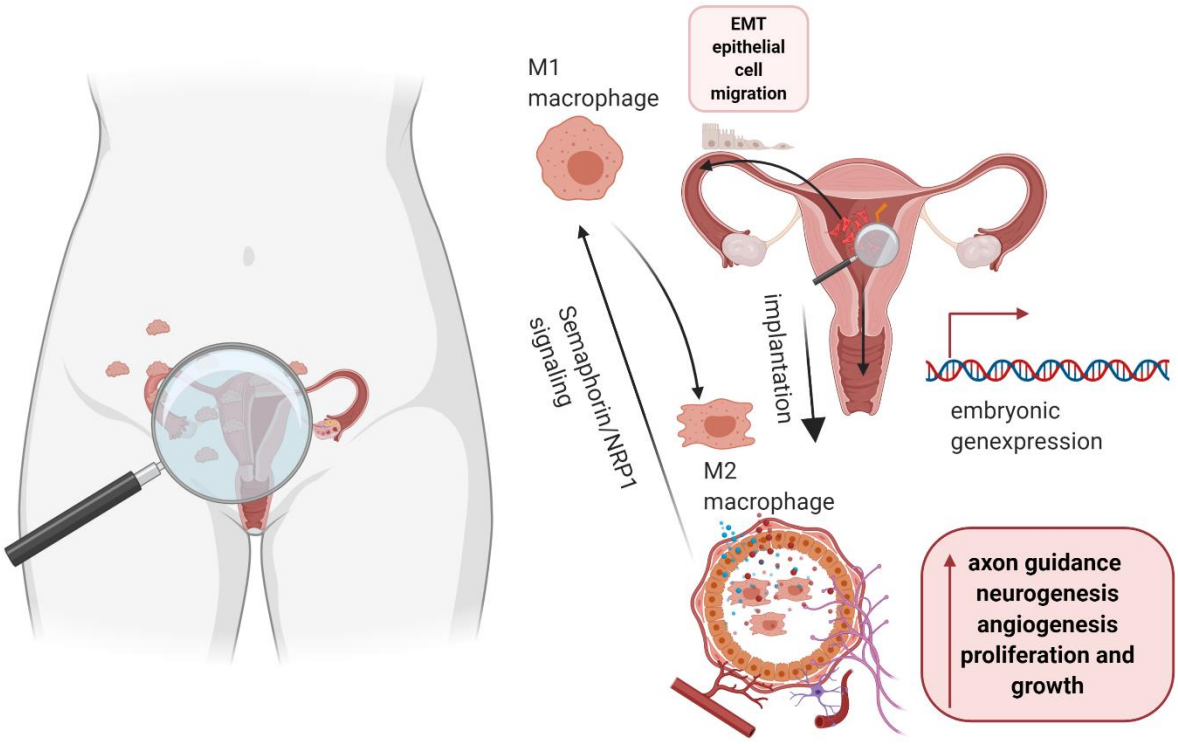


Figure 47 hypothesis of endometriosis development

Somehow whatever mysterious reason, endometriotic cells must have the ability preserved, since healthy endometrial cells (THESC) don't respond to TNFα in this way. Further investigation must be done, including proteomic analysis of endometriotic lesions as well as developing a cell culture (*163*)macrophage model of endometriotic like macrophages , since

primary macrophage collection and culture is a very complex performance (*164*). Even more, epithelial primary cell culture is very hard, which makes it hard to develop cell culture model conditions. Collection of patient sample is hard, since many women don't have a confirmed diagnosis due to lack of knowledge and since surgery is still the only way to diagnose endometriosis properly (1,4,6). Therefore, new techniques must be developed using co-culture of primary stromal endometriosis cells and epithelial cells. Proteomic analysis of menstrual blood could give insight into changes of endometrial cells, since 10% of menstrual fluid is retrograde into the pelvic area and more endometriosis develops. (*165*). All in all, analysis of endometriosis cell line 12-Z differed from healthy endometrial cell line THESC, and featured interesting findings upon TNFα stimulation compared to IL1β. Although these in vitro experiments are not able to completely cover the complexity of the in vivo situation to its full extend it provides valuable insight into the ongoing molecular mechanisms occurring during inflammatory stimulation. Therefore, this thesis provides the foundation for further investigations with primary patients' material.

7.4 Conclusion:

Endometriosis is a chronic inflammatory disease featuring multiple pathologic abnormalities, like neuroangiogenesis and modulation of macrophage functions. Inflammatory cytokines play a crucial role in establishment and progression of the disease.(2) Two of the main cytokines involved, IL1β and TNFα and their characteristic inflammatory behavior upon endometriotic epithelial cell line 12-Z and healthy endometrial cell line THESC, were characterized. Certainly, in vivo, TNFα and IL1β aren't the only cytokines involved in pathogenesis of endometriosis, therefore further investigation using more cytokines and other involved factors must be performed. Even the synergistic effect of IL1β and TNFα should be investigated using proteomic and multi-omics analysis. Proteomic analysis, revealed interesting properties of 12-Z endometriotic cells featuring neuronal developmental signaling and neuroangiogenic activity, in comparison to THESC cell line. Furthermore, possible embryonic neuronal developmental signaling was found upregulated in endometriotic cell line 12-Z.

Since endometriosis is a hormone dependent disease, hormonal interactions, especially estrogen signaling and the synergistic effect of cytokine signaling in neuroangiogenesis must be investigated using multi-omics approaches, like proteomic, phosphoproteomics, Eicosadomics and metabolomics. Metabolomic experiments using macrophage cell lines , in co-culture with endometriotic cells might give insight in macrophage reprogramming and sustainment of neurogenesis activity. Primary tissue analysis should be done using multi-omics approaches. Investigating angiogenic potential and possible dormant embryonic genexpression patterns, menstrual blood collection and analysis might be interesting. Various conditions and signaling cascades result in pathologic endometriosis appearances, but the cause remains still unknown. It is a debilitating disease, featuring enormous pain levels due to aberrant innervation and neuropathic inflammation. (49,51) Endometriosis, is a very complex and still incompletely understood disease, with a desperate need for a therapy. Hopefully, endometriosis research will expand and unravel these burning issues.

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