

AMERICAN UNIVERSITY OF BEIRUT

IDENTIFYING NOVEL SIGNALING MECHANISTIC
PATHWAYS UNDERLYING THE PATHOGENESIS OF
ENDOMETRIOSIS

by
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submitted in partial fulfillment of the requirements
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Approved by:

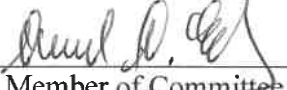
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ABSTRACT OF THE THESIS OF

Lama Issam Assaf

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Title: Identifying Novel Signaling Mechanistic Pathways Underlying the Pathogenesis of Endometriosis

Background: Endometriosis is the growth of endometrial tissue outside the uterine cavity usually in the ovaries, fallopian tube, and the pelvic cavity. It affects 1 in 10 women which is approximately 176 million women worldwide. Endometriosis is associated with severe pelvic pain, infertility, dysmenorrhea, and dyspareunia. The gold standard for confirmatory diagnosis is through laparoscopy which is not practical as a first diagnostic tool. Defining the molecular etiology of endometriosis is remarkably challenging for improving women's quality of life. Unfortunately, the pathophysiology of endometriosis remains to be elucidated. CYP4A and its metabolites, 20-HETE, is well known in its role in inflammation and angiogenesis. Further on, mTOR-signaling pathway is well described to be associated with cellular proliferation as well as organ injury through affecting myriads of pathways including inflammatory pathway. Recent research suggests that disrupted kinase signaling pathways and oxidative stress may play a role in proliferation and survival of endometrial cells outside their niches. Furthermore, our lab has previously shown that ROS, NADPH oxidase as well as NOX1 and NOX4 are upregulated in endometriosis.

Aim: In this study, we aim to investigate the role of AMPK/CYP4A/mTOR signaling axes in inducing the proliferation of endometriotic implants.

Methods: Ex vivo experiments were conducted on anonymous endometriotic tissues collected from women that underwent laparoscopy. Moreover, mTOR, Raptor, AMPK TGF- β and inflammatory cytokines (IL-6, IL-8 and TNF- α) expressions were assessed by PCR and CYP4A expression by western blot. In addition, HPLC was used to measure 20-HETE levels. Histological analysis was also performed.

Results: Endometriosis is associated with increased fibrogenesis and inflammatory markers. we postulate that endometrial injury is linked to inactivation of AMPK while mTOR is hyper-regulated. Furthermore, there was significant increase in CYP4A and 20-HETE production that leads to an increase in ROS production.

Conclusion. Collectively, our results display the role of AMPK/CYP4A/mTOR signaling axes in the pathogenesis of endometriosis. Therefore, targeting this pathway could be a potential therapeutic approach for the treatment of endometriosis.

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ABBREVIATIONS

AMPK	Adenosine Monophosphate-activated Protein Kinase
mTOR	Mammalian Target of Rapamycin
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
IL	Interleukin
ESC	Endometriotic stromal cells
PGE2	Prostaglandin E2
stAR	Steroidogenic acute regulatory protein
CREB	cAMP response element binding protein
CRTC2	CREB regulated transcription coactivator 2
Raptor	Regulatory-associated Protein of mTOR
mLST8	Mammalian Lethal with Sec13 protein 8
PRAS40	Proline Rich AKT Substrate
Deptor	DEP-domain-containing mTOR Interacting Protein
PIP2	Phosphatidylinositol-4,5-Bisphosphate
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
Rheb	Ras Homolog Enriched in Brain
FOXO1	Forkhead box protein O1
EBP1	ErbB3 Binding Protein 1
ROS	Reactive Oxygen Species
HIF-1 α	Hypoxia-Inducible Factor 1-alpha
VEGF	Vascular Endothelial Growth Factor

ER β	Estrogen Receptor- β
MMP	Matrix Metalloproteinases
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX	NADPH Oxidase
COX-2	Cyclooxygenase-2
CYPs	Cytochromes P450
TGF- β	Transforming Growth Factor β
TNF- α	Tumor Necrosis Factor α
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
JAK	Janus Kinase
STAT	Signal Transducer and Transcription Activators
MAPK	Mitogen-Activated Protein Kinase

CHAPTER 1

INTRODUCTION

1.1. Definition

Endometriosis is the presence of endometrial tissue outside the uterine cavity usually in the ovaries, fallopian tube, and the pelvic cavity. Recent studies also showed the presence of endometrial tissue in the lungs, umbilical and neuron (Chamié, Ribeiro et al. 2018). It affects 1 in 10 women which is approximately 176 million women worldwide (Parasar, Ozcan et al. 2017). It is an estrogen-dependent chronic inflammatory condition that affects women in their reproductive period and associated with chronic pelvic pain, dysmenorrhea (painful period), dyspareunia (painful sex), Mittelschmerz (painful ovulation), bowel or bladder associated pain or symptoms, infertility and collapsed lungs in case of lung endometriosis (Sonavane, Kantawala et al. 2011). Endometriotic lesions have been classified into peritoneal implants, ovarian cysts (endometrioma) and deep infiltrating endometriosis (which can individually involve and infiltrate the parametria, douglas pouch, anterior rectal wall, posterior vaginal fornix, vesico-uterine pouch, bladder detrusor, ureters, and sigmoid colon) (Sonavane, Kantawala et al. 2011). Endometriomas have a unique physiological characteristic among benign ovarian cysts because they are formed by an extraovarian hematoma, surrounded by duplicated ovarian parenchyma according to the invagination theory. This finding explains why surgical ‘enucleation’ of the pseudocapsule implies removal of part of the gonadal cortex, follicle loss and reduction of the ovarian reserve (Prentice 2001).

Endometriosis

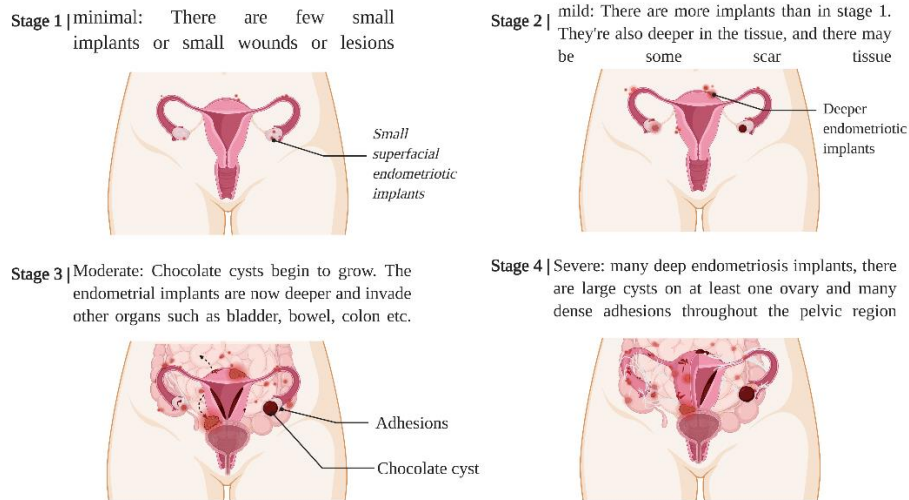


Figure 1. Stages of Endometriosis Progression Assaf L. (created using Biorender)

1.2. Diagnosis

The gold standard for confirmatory diagnosis for endometriosis is laparoscopy. The issue with laparoscopy is that it is not practical as a definitive diagnostic tool (Bafort, Beebeejaun et al. 2020). Since deep endometriotic implants might be hidden under extensive adhesions, or simply may not be conspicuous because of their location. Thus, surgical exploration as a diagnostic technique becomes sophisticated (Bafort, Beebeejaun et al. 2020). Presurgical mapping of the endometriotic lesions is critical towards ensuring success in such ventures. Currently, ultrasound is favored for the assessment of both endometriomas as well as deep pelvic endometriosis (Dinh, Leonardi et al. 2020). However, even with adequate bowel preparation and use of high-frequency probes, transvaginal ultrasound has important limitations. This is due to its relatively small field-of-view and operator dependency (Imanaka, Maruyama et al. 2020). Another imaging technique, magnetic resonance imaging (MRI), is being progressively used for the evaluation of endometriosis, with reported sensitivity 69-92%

and specificity values 75-98% (Foti, Farina et al. 2018). Most physicians advocate MRI as an adjunctive tool in cases of ultrasound-indeterminate findings, possible ureteral involvement, and presurgical mapping (Sonavane, Kantawala et al. 2011). However, negative tests do not definitively rule out the diagnosis of endometriosis (Imanaka, Maruyama et al. 2020). Peritoneal lesions can be simply too small to be picked up on MRI, but their presence can cause substantial peritoneal irritation; this is while keeping in mind that the size and number of lesions do not necessarily correlate with the amount of pain patients experience. Lesions of small number and size can result in debilitating pain, even if ALL diagnostic studies, such as imaging modalities, are negative. That's why laparoscopy remains the definitive diagnostic tool and the use of non-invasive tests should only be undertaken in a research setting. Because of the lack of reliably sensitive diagnostic tools, the average diagnostic delay for endometriosis is, on average, 7-12 years (Sonavane, Kantawala et al. 2011). Over 200 patients with a confirmed diagnosis for endometriosis underwent a survey by National Endometriosis Society. The survey highlighted that 32% consulted one specialist before being referred to a gynecologist, 25% consulted two other specialists before seeing a gynecologist and over half the patients had been told that there was nothing wrong. This engenders dismay in patients and delays effective treatment (Juhasz-Böss, Laschke et al. 2014)

1.3. Treatment

Treatment for endometriosis includes management of symptoms, surgical diagnosis, and surgical removal of disease. For management of symptoms, analgesics, such as non-steroidal anti-inflammatory drugs are often used (Brown, Crawford et al. 2017). Hormonal birth control medications are also commonly given continuously to

stop menstruation. This includes combined birth control pills (Jensen, Schlaff et al. 2018), progestin only birth control pills (Casper 2017), progesterone containing intrauterine devices (Mirena) (Chowdary, Maher et al. 2019) , injectable progesterone (Gezer and Oral 2015), and implanted progesterone (Gezer and Oral 2015). A more extreme symptom management option is artificial menopause, where hormonal medications, such as GnRH agonists like Lupron (Dlugi, Miller et al. 1990), GnRH antagonists suchlike Orilissa, and androgens like Danazol, are administered (2010). However, Danazol is no longer recommended because of its virilizing effects. As a whole, symptomatic medication does not stop the progression of the disease, does not treat it, and symptoms reoccur once treatment is stopped (Ferrero, Camerini et al. 2011, Medicine 2014, Shen, Ma et al. 2017, Vercellini, Viganò et al. 2018).

As for treatment, surgery is the method of choice. Different surgical procedures include ablation, excision, hysterectomy, and ovariectomy. However, ablation assures only short-term relief and most of the disease is left behind. Neither hysterectomy nor ovariectomy treat nor stop the progression of the disease unless the lesions in questions are on the structure of the uterus that was removed or in the ovary. The gold standard is excision: the complete removal of all the disease at its roots (Moawad, Arkerson et al. 2018). Although remission is low, it may not address endometriosis in certain places such as around the bowel, ureters, or diaphragm (Redwine and Hopton 2018). In addition, complicated excision surgery requires an endometriosis surgeon of high experience and skill, along with another specialized surgeon to remove lesions in case they are present on the bowel, lung, sciatic nerve, or other sensitive locations (Carugno 2018).

1.4. Pathophysiology

The etiology of endometriosis remains elusive. There are many theories to explain the pathophysiology of endometriosis. There are Sampson's Implantation Theory (Batt 2011), embryonic rest theory (Sasson and Taylor 2008), lymphovascular metastasis theory (Jerman, Anderson et al. 2020) and Mayer's coelomic metaplasia theory (Mulgund, Doshi et al. 2015). The embryonic rest theory states that during embryonic stage development, certain stimuli cause the differentiation of rest cells of mullerian origin into endometrial cells. These endometrial cells that have developed in the uterus, develop also in the abdomen, and become activated during puberty same as the endometrium. The lymphovascular metastasis theory postulates that like cancer, endometrial cells spread through the blood and lymphatic vessels since there is de novo formation of one third of the microvascular endothelium of ectopic endometrial tissue from endothelial progenitor cells. The coelomic metaplasia theory suggests that a factor found in menstrual blood causes the metaplastic transformation of the epithelial lining in the peritoneal cavity into endometrial cells (Mulgund, Doshi et al. 2015). However, the most acceptable one is the theory of implantation which conveys that endometriosis is the result of retrograde menstruation. Endometrial fragments are refluxed through the fallopian tubes, possibly by a pressure gradient from uterine contractions (Vercellini, Viganò et al. 2014). These regurgitated endometrial cells located outside the uterus stimulate the infiltration of immune cells (such as, macrophages and mast cells) into the lesions, secrete inflammatory mediators (such as, proinflammatory cytokines, chemokines, and nerve growth factor), consequently resulting in an inflammatory peritoneal microenvironment (Chapron, Marcellin et al. 2019). Nevertheless, Retrograde menstruation occurs in 76% to 90% of women and only approximately 10%

suffer from endometriosis. Besides, it raises further question about how the endometrioma migrates and even survives in such niches (Seli, Berkkanoglu et al. 2003).

1.4.1. Kinase Signaling

1.4.1.1. Adenosine Monophosphate-activated Protein Kinase (AMPK)

1.4.1.1.1. Structure and Signaling Pathway

AMPK is a heterotrimeric protein complex consisting of 3 subunits α , β , and γ subunits that make the functional enzyme (Mihaylova and Shaw 2011). There are 2 or 3 genes encoding each subunit and by alternative splicing, gives rise to 12 possible heterotrimeric combinations. Thus, increasing the potential diversity. Each subunit codes for specific role. For example The γ subunit includes four particular Cystathionine beta synthase (CBS) domains, giving AMPK its ability to sensitively detecting shifts in the AMP:ATP ratio (Mihaylova and Shaw 2011). Further on, AMPK heterotrimer are differentially expressed based on biological context and tissue type. Current model states that AMP/ADP binds to CBS domain in the gamma subunit. Then the α subunit is subsequently phosphorylated on threonine-172, which amplifies its kinase activity. This leads to conformational changes that allosterically activates AMPK and renders phosphorylated-Thr172 unavailable for inhibitory dephosphorylation (Shirwany and Zou 2014).

1.4.1.1.2. Role of AMPK

Recent studies show that AMPK serves as novel therapeutic target for the treatment of type-1 and type-2 diabetes since pharmacological activation of AMPK improves blood glucose homeostasis, lipid profile and blood pressure in insulin-resistant rodents (Eid, Ford et al. 2010). Also, it is evident that AMPK dysregulation

contributes to cancer (Li, Saud et al. 2015). In response to a decrease in cellular adenosine triphosphate (ATP), as detected by the ratio of ATP with its dephosphorylated precursors adenosine diphosphate (ADP) or adenosine monophosphate (AMP), AMPK plays a role in cellular energy homeostasis (Mihaylova and Shaw 2011). In fact, AMPK is an energy-sensing factor which rewires metabolism and maintains redox balance. It acts to initiate catabolic processes while concomitantly heavily inhibiting energy dependent anabolic pathways. The interference with the catabolic generation of ATP (e.g., glucose deprivation, hypoxia, and ischemia) or accelerated ATP consumption (e.g., muscle contraction), increases cellular ADP: ATP and AMP:ATP ratios. AMPK becomes activated and regulates energy hemostasis by switching on catabolic pathways that generate ATP, while switching off anabolic pathways that consume ATP (Mihaylova and Shaw 2011). AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species (Rabinovitch, Samborska et al. 2017), Stimulates mitochondrial biogenesis (Herzig and Shaw 2018), regulates autophagy (Li and Chen 2019) rewires myriads of metabolic pathways like glucose, lipids, and protein metabolism (He, Zhou et al. 2017) and balance redox reaction (Eid, Ford et al. 2010). Therefore, alteration in AMPK pathway leads to increased reactive oxygen species (Eid, Ford et al. 2010).

1.4.1.1.3. Targeting AMPK as a Therapeutic Approach

Many experiments were conducted to activate AMPK pathway as therapeutic approach for treatment of endometriosis. Metformin, antidiabetic drug, causes AMPK activation. One study investigated the effects of metformin on experimentally induced endometriosis in a rat model (Oner, Ozcelik et al. 2010). The experiment was designed as the following, 38 rats were randomly divided into four groups and administered

different doses of metformin. All rats continued to receive the treatment for 4 weeks and then were sacrificed to assess the size of implants and scores of adhesions. As a result metformin reduced the size of the endometriotic implant and severity of adhesions (Oner, Ozcelik et al. 2010). Another study demonstrated whether metformin may be effective for the treatment of endometriosis. Endometrial stromal cells (ESCs) derived from ovarian endometriomas were cultured with various concentrations of metformin (Takemura, Osuga et al. 2007). Then interleukin-8 (IL-8) production, mRNA expression, aromatase activity, and 5-bromo-2'-deoxyuridine incorporation in ESCs were measured. Results verified that metformin inhibits interleukin-1 β -induced IL-8 production, aromatase activation, and proliferation of ESCs (Takemura, Osuga et al. 2007). However, the drawback regarding these studies is that it did not emphasize the exact mechanism through which metformin affects the endometriotic implants. Steroid acute regulatory protein (stAR) is an enzyme responsible for controlling the rate-limiting step of steroid biosynthesis by regulating the delivery of cholesterol to the inner membrane of mitochondria for cholesterol conversion into estrogen (Tsai, Wu et al. 2001). stAR is regulated by cyclic AMP response element binding protein (CREB) and CREB-regulated transcription coactivator (CRTC2) (Smith, Huang et al. 2020). It is believed that ectopic endometrial implants are capable of de novo synthesis of estrogen due to altered expression of stAR (Tsai, Wu et al. 2001). A study investigated the molecular and cellular mechanism by which metformin regulates stAR expression in human endometriotic stromal cells (Xu, Zeng et al. 2014). Endometriotic stromal cells derived from ovarian endometriomas were cultured with metformin and prostaglandin E2 (PGE2). Pregnenolone, progesterone and estrogen production, StAR, AMPK, CREB, and CRTC2 protein expressions were measured.

CRTC2 translocation and its association with CREB were assessed by coimmunoprecipitation assay. Results showed that StAR mRNA levels in ESCs are 264 times higher than those in endometrial cells. Because PGE2 induces CRTC2 translocation and enhances its association with CREB to form a transcription complex that binds to the StAR promoter region, Metformin downregulated the StAR mRNA expression via disrupting CREB-CRTC2 Complex Formation. Therefore, Metformin prevents the nuclear translocation of CRTC2 by increasing AMP-activated protein kinase phosphorylation. This inhibits transcription of StAR by disrupting formation of the CREB-CRTC2 complex, involved in activation of the StAR promoter cAMP response element (Xu, Zeng et al. 2014). Not only does metformin activates AMPK but also inhibits mTORC1 (Zhang, Jiang et al. 2014). Metformin acts on v-ATPase and enhances the translocation of AXIN/LKB1 onto the surface of lysosome to form complex with v-ATPase-Regulator, consequently AMPK activation (Zhang, Jiang et al. 2014). Because of the recruitment of AXIN, the v-ATPase-Regulator complex dissociates Raptor and mTOR. Thus, turning off the activity of mTORC1 (Zhang, Jiang et al. 2014).

1.4.1.2. Mammalian target of rapamycin (mTOR)

1.4.1.2.1. Structure and Signaling Pathway

mTOR is a conserved serine/threonine kinase of the phosphatidylinositol kinase-related kinase family that drives myriads of cellular functions such as cell proliferation (Ryskalin, Lazzeri et al. 2017), migration (Zhou and Huang 2011), adhesion (Chen, Xu et al. 2015), metabolism (Zhang, Jiang et al. 2014), and invasion (Zhou and Huang 2011). mTOR exists as 2 complexes: mTOR complex 1 (mTORC1) and complex 2

(mTORC2). Both mTOR complexes share the mTOR catalytic subunit, mammalian lethal with sec-13 protein 8 (mLST8), Tti1/Tel2 complex, and DEP domain-containing mTOR-interacting protein (DEPTOR). However, mTOR complex 1, the most extensively studied complex, is made up of the regulatory-associated protein of mammalian target of rapamycin (Raptor), and proline-rich Akt substrate (PRAS40) specifically (Dazert and Hall 2011). Downstream of mTORC1 there are p70S6 kinase/S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1), which induce its effect on protein synthesis and cell growth. Since mTORC1 mainly influences protein synthesis by balancing cellular anabolism and catabolism, it dictates cell size (Dazert and Hall 2011).

The activation/inactivation of mTORC1 requires the integration of several signals such as nutrients (glucose and amino acids), energy in the form of ATP, growth factors such as cytokines, and hormones such as estrogen (Dazert and Hall 2011, Maruani, Spiegel et al. 2012). It is mainly dependent on small Ras homolog enriched in brain (Rheb) proteins, a monomeric ubiquitously expressed GTPase. When it's bound to GTP, Rheb is activated and subsequently activates downstream effectors. It is also deactivated when it is bound to GDP. Through its effect via mTOR/S6K and depending on the intracellular GTP/GDP ratio, GTPase can function as a molecular switch for various cellular processes including amino acid uptake, autophagy, metabolism, cell cycle, and cell growth (Laplante and Sabatini 2009). Some studies show that Rheb activates mTORC1 directly, through interaction with mLST8, the kinase domain of mTOR, as well as Raptor. To emphasize, receptor tyrosine kinases (RTK) are activated upon ligand binding (i.e., growth factors) and subsequently phosphorylate Phosphatidylinositol 3-kinase (PI3K) directly or indirectly through the interaction of

multiple adapter proteins. PI3K converts intracellular phosphatidylinositol-4,5-bisphosphate (PIP₂) (PIP₂) to phosphatidylinositol-3,4,5-Triphosphate (PIP₃) (Laplante and Sabatini 2009). PIP₃ in turn interacts with and recruits many signalling proteins into the plasma membrane including the protein kinase B (Akt), mainly through binding to the pleckstrin homology (PH) domain of these proteins. Once activated downstream of PIP₃, Akt inhibits tuberous sclerosis gene products (TSC complex). TSC complex, made up of TSC complex subunits 1 and 2 (TSC1 and TSC2), in its active form converts Rheb into its inactive GDP-bound state halting mTORC1 activity. When TSC2 is phosphorylated by kinases such as Akt it dissociates from the complex rendering it inactive, which allows the GTP-bound Rheb to activate mTORC1. Therefore, leading to mTORC1 activation (Laplante and Sabatini 2009). Further on, amino acids induce the migration of the mTOR complex from the cytosol by a Ras-related GTPase (Rag)-dependent system through extracellular signal-related kinase (ERK) (Shimobayashi and Hall 2016). Once activated, mTORC1 stimulates the biosynthesis of three major classes of macromolecules: proteins, lipids and nucleic acids and their precursors as well as the production of ATP and reducing factors (NADPH) (Shimobayashi and Hall 2016). Once mTORC1 is activated it activates myriads of downstream proteins. It activates S6K1 and 4E-BP1, leading to protein synthesis and cell growth. Moreover, activates HIF- α , master angiogenic switch, through its well-known vascular endothelial growth factor (VEGF) activation (Karar and Maity 2011). Additionally, it activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) pathway, well known in cytokines production and inflammation (Weichhart, Costantino et al. 2008).

1.4.1.2.2. Role of mTOR Signaling Pathway

The mTOR pathway is a well-characterized focal point in studies of diseases like cancer, diabetes, and Alzheimer's (Mroueh, Noureldein et al. 2019, Tian, Li et al. 2019). Moreover, it is well known for its role in cell growth (Ryskalin, Lazzeri et al. 2017), proliferation (Ryskalin, Lazzeri et al. 2017), and migration (Zhou and Huang 2011).

The most critical function of mTOR is regulating metabolism by integrating signals from growth factors, nutrients, oxygen, and energy status (Dazert and Hall 2011, Maruani, Spiegel et al. 2012). Additionally, mTOR is well known to promote cell proliferation by its downstream effectors, S6K and 4EBP (Dazert and Hall 2011). Also, mTOR promotes angiogenesis and inflammation via activating its downstream effectors, HIF- α and NF- κ B respectively (Weichhart, Costantino et al. 2008, Karar and Maity 2011).

mTOR signaling pathway plays an essential role in regulating female reproduction, which has been demonstrated based on data from genetic, pharmacological, and clinical studies (Guo and Yu 2019). It is involved in folliculogenesis, puberty onset and fertility (Guo and Yu 2019). For instance, disruption of TSC2, negative mTORC1 regulator, activates primordial follicle (Adhikari, Flohr et al. 2009). Thus, mTORC1 signaling is an important mechanism of primordial follicle activation. Moreover, HIF- α is not only angiogenic switch but also necessary for follicular differentiation of granulosa cells via follicular stimulating hormone (FSH). Knowing that HIF- α is downstream target of mTORC1, thus mTOR activation is implicated in follicular development of granulosa cells leading to follicular differentiation to a preovulatory phenotype (Alam, Maizels et al. 2004). Moreover, mTOR signaling plays pleiotropic roles in the process that occurs

in the ovary, including ovarian reserve, follicle development, oocyte meiotic maturation, ovarian aging, proliferation and steroidogenesis of ovarian somatic cells (Liu, Liao et al. 2016). A study has shown that mTORC1 contributes to maintenance of oocyte genome integrity, oocyte gene expression, meiosis, and preimplantation developmental competence (Guo, Zhang et al. 2018). mTORC1 signaling activation through S6K1 and 4EBP1, is highly regulated to ensure oocyte meiotic maturation that encompasses activation of translation and increase in protein synthesis, nuclear envelope breakdown (NEBD), chromatin condensation, first meiotic spindle formation (metaphase I, MI), and the first polar body extrusion (metaphase II, MII) (Kogasaka, Hoshino et al. 2013). Additionally, mTOR signaling also participates in steroidogenesis. In human inhibition of mTORC1 by rapamycin, decreases progesterone production in human granulosa lutein cells (Moravek, Shang et al. 2016).

The endometrium undergoes cyclical and rhythmic changes under the influence of complex autocrine, paracrine, and endocrine signaling. These processes involve cell proliferation, differentiation, apoptosis, autophagy, and decidualization, in which mTOR signaling is well established to play role in (Roberti, Higa et al. 2018). Estradiol-17 β (E2) and P4 are the most important factors orchestrating cell division and differentiation of the endometrium. It is well known that E2 regulates protein synthesis and DNA synthesis in uterine epithelial cells through the phospho-kinase C (PKC)/ERK/mTOR pathway, which finally manipulates cell proliferation (Wang, Zhu et al. 2015). Via autophagy induction, mTOR signaling stimulates human endometrial stromal cell (ESC) apoptosis (Choi, Jo et al. 2014). Decidualization, the functional and morphological changes that occur within the endometrium to form the decidual lining into which the blastocyst implants, is highly affected via the PI3K-Akt-mTOR signaling

pathways in mouse ESCs and human ESCs. Because of the vital roles of mTOR in modulating the endometrium, it renders the endometrium suitable for embryo development by influencing endometrial receptivity (Zhang, Fu et al. 2016).

Since mTOR signaling is associated in many processes such as cell proliferation, inflammation, metabolism and reproduction, modulation in this pathway has been explored in preclinical studies (Guo and Yu 2019). For that, preclinical studies convey the possibility of mTOR in the etiology of gynecological diseases such as endometriosis.

1.4.1.2.3. Targeting mTOR as a Therapeutic Approach

It is suggested that AKT promotes survival of endometriotic cells. Leconte et al. (Leconte, Nicco et al. 2011) reported that treatment of endometriotic cells with the mTOR inhibitor, temsirolimus, resulted in decreased proliferation and viability both in vitro and in mouse models. The expression of Akt, NF- κ B, antiapoptotic factors B-cell lymphoma-extra-large and X-linked inhibitor of apoptosis protein in endometriotic cells was inhibited by sesquiterpene lactone costunolide, an mTOR pathway inhibitor (Kim, Yang et al. 2011). Consequently, these studies support the role of AKT in promoting proliferation and survival of endometriotic cells. MK-2206, an AKT inhibitor, attenuates proliferation of human endometriotic stromal cells in culture compared with disease-free endometrial stromal. This compound reduced the levels of a target protein p(S256)-foxO1 and decreased the viability of cells from women both with and without endometriosis (Matsuzaki, Pouly et al. 2018). Leflunomide, a tyrosine kinase inhibitor, blocks NF- κ B transcription and reduced endometriotic cell proliferation in vitro. Temsirolimus, a specific mTOR inhibitor, blocked proliferation of endometriotic cell in vitro and in a heterologous nude mouse model (Leconte, Nicco et al. 2011). Yang Cao

et al (Cao, Ye et al. 2017) investigated the effect of ginsenoside Rg3 on endometrial cells. Real-time PCR results showed that the mRNA expression level of VEGF, Akt, and mTOR in the ectopic endometrium was reduced. Immunohistochemical and Western Blotting assays confirmed that the expression of VEGF, p-Akt, and p-mTOR was down-regulated in ginsenoside Rg3 -treated lesions. This proved that ginsenoside Rg3 plays a role by halting VEGFR-2-mediated PI3K/Akt/mTOR signaling pathway. Thus, blocking angiogenesis and promoting the apoptosis of ectopic endometrial cells.

Because endometriosis shows inadequate response to progesterone in both the eutopic and ectopic endometrial cells and tissue, it is considered as progesterone resistant. Genes for the expression of progesterone target are blunted (Kao, Germeyer et al. 2003, Osteen, Bruner-Tran et al. 2005). Progesterone receptor levels are low in endometriotic tissues and cells (Attia, Zeitoun et al. 2000). Hence, inefficient decidualization and lesions remain throughout the cycle.

The drawback regarding targeting mTOR signaling pathway is the unwanted side effects. Despite the promising results shown by the listed compounds, these classes of drugs are suggested to be teratogenic. Yet temsirolimus, which has shown promise in reducing endometriotic lesions in vitro and animal models, is currently approved for treatment of renal cell carcinoma. Through this use, the class-specific toxicities of these drugs are emerging. Adverse effects commonly include an impact on the hematological, pulmonary, and dermatological systems (Eisen, Sternberg et al. 2012). However, by carefully monitoring the patient, the side effects withdraw after cessation of the therapy. mTOR signaling disruption is implicated in many human cancers. Targeting this pathway in endometriosis showed reduction in endometrial cells in vivo and invitro. Besides, the unique anatomy of the female reproductive system, attains accessibility for

localized drug delivery that would minimize the systemic concentrations and, as a result, reduces side effects of drugs. Therefore, mTOR pathway inhibitors represent a potentially new approach in the treatment of endometriosis. Nevertheless, the side effects should be carefully monitored and delineated.

1.4.1.3. Crosstalk between mTOR and AMPK

AMPK and mTOR pathways are interlinked, opposing signaling pathways involved in sensing availability of nutrients and energy and regulation of cell growth (Hindupur, González et al. 2015). AMPK (Yin, or the “dark side”) is switched on during energy stress and inhibits cell growth, while TOR (Yang, or the “bright side”) is switched on by nutrient abundance and promotes cell growth (Hindupur, González et al. 2015). Crosstalk between mTOR axis, and the AMPK axis was discovered because AMPK regulates S6K and 4EBP, the main downstream effectors through which mTORC1 acts, suggesting that these two axes converge at some point. Akt, also referred to as protein kinase B (PKB), is one of the common points between the two axes (Wang, Shen et al. 2019).

Moreover, studying TSC complex gave insight about the crosstalk between the two axes. As discussed earlier, the tuberous sclerosis complex (TSC) is a stable heterodimer made of TSC1 (hamartin) and TSC2 (tuberin). This complex is directly targeted by AMPK and known to be mediator between AMPK’s effect on the mTOR pathway. In fact, AMPK phosphorylates TSC2 on two different positions T1227 and S134. Thereby inhibiting mTORC1 by enhancing stability of TSC complex (Inoki, Zhu et al. 2003). Indeed, cells bearing mutations in the AMPK phosphorylation sites of TSC2 do not show any size reduction upon ATP depletion. Also, in cells lacking TSC2,

ATP depletion no longer inactivates mTORC1, leading to the increased phosphorylation of S6K1 and 4EBP. This suggests that a low energy profile in the cell activates AMPK, which in turn inactivates mTORC1 by enhancing the action of TSC.

1.4.2. Oxidative Stress

It is widely accepted nowadays that oxidative stress contributes to the pathophysiology of endometriosis. Reactive oxygen species are inflammatory mediators known to modulate cell signaling and homeostasis. However, when imbalance occurs between ROS and antioxidants, ROS cause irreversible damage to DNA that could eventually contribute to cell death. Oxidative stress has been implicated in numerous diseases such as diabetes (Eid, Massaad et al. 2016), cardiovascular diseases (Dhalla, Temsah et al. 2000, Schisterman, Faraggi et al. 2001), chronic kidney diseases (Aghadavod, Khodadadi et al. 2016), and male and female infertility (Agarwal, Gupta et al. 2005, Menezo, Silvestris et al. 2016).

1.4.2.1. Role of CYP Enzymes in ROS Metabolism and Endometriosis

The course of inflammation is coupled with the activation of the coagulation system, increased perfusion, and vascular permeability, as well as the production of inflammatory mediators, including arachidonic acid (AA) and linoleic acid (LA) derivatives. AA is the most abundant fatty acid that is also biosynthesized from linoleic acid (Brücher and Jamall 2019). Under the influence of cyclooxygenase (COX) enzymes, AA is transformed into prostaglandins (PG), thromboxane, and leukotrienes (Sonnweber, Pizzini et al. 2018, Brücher and Jamall 2019). Also, via cytochrome P450 (CYP) enzyme activity, AA is metabolized into epoxyeicosatrienoic acids (EETs) and

20-hydroxyeicosatetraenoic acid (20-HETE) (Capdevila, Wang et al. 2015, Brücher and Jamall 2019). As part of normal physiology of the female body, inflammation is a normal process of menstrual cycles, embryo implantation, course of pregnancy, and childbirth. However, excessive activation of inflammation or quenching reaction deficit, results in the development of disorders and uncontrolled inflammation leading to in this case to endometriosis. As CYP enzymes metabolize their substrates, they produce ROS. This can increase cellular damage via increase in protein, nucleic acid, and lipid modifications. These products contribute to numerous human pathologies as its already observed in the role of CYP in cancer progression (Johnson, Edson et al. 2015).

In endometriosis a study showed that downregulation of eicosapentaenoic acid (EPA) to AA ratio (EPA/AA) commensurate with the severity of Endometriosis (Khanaki, Nouri et al. 2012). Moreover, another study denoted that AA concentration was remarkably higher in women suffering from endometriosis than in control (Li, Guan et al. 2018). Since endometriosis is estrogen dependent disease, several genes are involved in oxidation metabolism of estrogen. Increased cyclooxygenase-2 (COX-2), which elevates levels of prostaglandin E₂, increases the levels of aromatase, an enzyme necessary for estrogen production (Wright, Hoffman et al. 2019). In a positive feedback loop estrogen then induces COX-2 expression (Tamura, Deb et al. 2004).

Some studies displayed the participation of HETE in endometriosis pathogenesis. For example, it has been found that inflammation expressed by the presence of 12-HETE differs in a manner relative to the endometrial cell location. 20-HETE, generated by CYP4A and CYP4F enzymes is a well-established proinflammatory mediator of the inflammatory cascade (Johnson, Edson et al. 2015, Brücher and Jamall 2019). 20-HETE has long been implicated in cellular proliferation ,

often invoking the participation of growth factors, such as VEGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Chen, Ackerman et al. 2014, Johnson, Edson et al. 2015). Additionally, 20-HETE induces oxidative stress by increasing NADPH (Dunn, Renic et al. 2008). It is also known for activating protein kinase C (PKC), which plays an important role in eukaryotic cell physiology, in particular signal transduction pathways (Kunduri, Mustafa et al. 2013). 20-HETE is known to activate NF- κ B pathway (Garcia, Shkolnik et al. 2016). Thus, 20-HETE affects numerous cellular responses including gene expression, protein secretion, cell proliferation, and the inflammatory response. However, little is known about its involvement in the physiological changes wrought by endometriosis.

1.4.2.2. Fibrosis

Fibrosis is a natural phenomenon critical for wound healing. However, when alteration occurs, it becomes causative agent of loss of functional organ tissue, resulting in diseases such as cirrhosis, Crohn's disease, and pulmonary fibrosis (Bataller and Brenner 2005, Li and Kuemmerle 2014). Fibrosis is predominantly characterized by excessive deposition of extracellular matrix (ECM), especially collagen. This excessive deposition results in modulated tissue and organ architecture that leads to dysfunction and ultimately pathology. Moreover, fibrosis occurs as part of epithelial-mesenchymal transition (EMT) in a variety of cancers wherein it is associated with adverse prognosis (Thiery, Acloque et al. 2009). Fibrosis appears as the framework for endometriosis-associated symptoms (pain and infertility) and disease manifestations (i.e. adhesions) (Vigano, Candiani et al. 2017). Fibrosis cause infertility because of lesions that are

associated with prolific fibrosis which entangles abdominal and pelvic organs into a highly distorted pelvis with concomitant multi-organ functional impairment (Vigano, Candiani et al. 2017). Hence, creating inflammatory environment unviable for implantation, and embryo development as well as occlusion of the tubal ostium compromises sperm passage. Also, fibrosis is linked to recurrence after conventional surgical treatment for the disease (Vercellini, Crosignani et al. 2009). The development of fibrosis in endometriotic lesions represents a complex phenomenon with underlying mechanisms yet to be fully clarified. Macrophages are main cellular components playing a key role in fibrosis development (Johan, Ingman et al. 2019). When macrophages are activated by IL-4, and phagocyte ectopic endometrial cells, they secrete IL-6, IL-8 and TGF- β . Therefore, through TGF- β 1/Smad3 signaling activation induce EMT and fibroblast to myofibroblast transition FMT (Duan, Liu et al. 2018). Further on, oxidative stress contribute to TGF- β signaling pathway activation, which then stimulates either SMAD-dependent or SMAD-independent pathways (e.g., phosphatidylinositol-3-kinase (PI3K), c-Jun N-terminal kinases (JNK)) (Liu and Desai 2015). Moreover, Increased TGF- β signaling also stimulates production of nitric oxide (NOX4)-generated ROS (Chan, Peshavariya et al. 2013), which further induces the transcriptional activities of pro-fibrotic genes such as collagen I (COL1), and NOX4. In addition, increase in NOX4 mediates cross-talk between other ROS-dependent signaling transduction pathways such as, NF κ B (Nakano, Nakajima et al. 2006). Together, ROS overproduction and TGF- signaling stimulate proliferation and trans differentiation of fibroblast cells into myofibroblasts, and excessive ECM deposition leading to fibrosis. Additionally mTORC1 regulates inflammatory cells such as macrophages (Byles, Covarrubias et al. 2013) and prominently produce TGF- β (Cheng and Hao 2017). In

contrast, some studies showed that TGF- β activates mTOR through phosphatidylinositol 3-kinase and Akt leading to the activation of mTOR direct regulators of translation initiation, S6 kinase 1 and eukaryotic initiation factor 4E-binding protein 1 (Lamouille and Derynck 2007).

1.4.3. Inflammation

Inflammation is a recognized factor contributing to reproductive dysfunction and pain. Ectopic endometrial tissues create several chronic inflammatory environments, particularly in the peritoneal cavity, ovaries, and uterus. Fertility is impaired due to chronic inflammation found in endometriosis. Studies have shown reduced ovarian response is associated with the increase in concentration of interleukin-1 β (IL-1 β), IL-8, IL-10, and tumor necrosis factor- α (TNF- α) in follicles adjacent to endometriomas (Opøien, Fedorcsak et al. 2013). Studies investigating peritoneal fluids of women with endometriosis showed elevation of estradiol, progesterone, monocyte chemoattractant protein (MCP)-1, TGF- β , VEGF, and proinflammatory cytokines such as interleukin (IL)-1, IL-6, and IL-8, and TNF- α , among others. Studies have shown that inflammatory mediators of the peritoneal fluid contribute to sperm DNA damage (Mansour, Aziz et al. 2009) and elevation in IL-6 levels inhibits sperm motility (Yoshida, Harada et al. 2004). Since ampulla of the fallopian tube and intrauterine environment are structurally exposed to peritoneal fluid, changes in the inflammatory factors negatively affects them. For example, prostaglandins generated from cytochrome p450, oxidative stress, and cytokines hinder implantation, embryo development and oocyte-sperm interactions (Chyra-Jach, Kaletka et al. 2018, Niringiyumukiza, Cai et al. 2018). Additionally chronic inflammation promotes a

proliferative and angiogenic environment that enhances endometriosis development and progression. Moreover, TNF- α , IL-6 and IL-8 are also involved in implantation, placentation, and pregnancy. The overexpression of TNF- α , IL-6 and IL-8 can impair follicular steroidogenesis, growth, and ovulation (Omere, Richardson et al. 2020), Hence, leading to infertility.

Ovarian endometriomas also called chocolate cyst modulates ovarian function through direct local effect and space occupation (Sanchez, Viganò et al. 2014). Cystic fluid inside chocolate cyst provides major source of proinflammatory cytokines (IL-6, IL-8), iron, reactive oxygen species (ROS), growth factors such as TGF- β , and matrix metalloproteases (MMPs) (Sanchez, Viganò et al. 2014). Because of the local inflammatory reactions found in the ovarian cortex surrounding endometriomas, this explains the increased oxidative stress observed there. This is also correlated with decrease oocyte quality and fertility (Kitajima, Defrère et al. 2011). Additionally structural alteration like the loss of ovarian stroma has a detrimental effect on folliculogenesis due to reduced blood supply to follicles and decreased growth factors secreted by stromal cells (Hsueh, Kawamura et al. 2015). Further on, abdominal bloating is under-recognized endometriosis symptom that could be as a result of high inflammation found in the bowel of women with endometriosis. (Habib, Centini et al. 2020).

CHAPTER 2

HYPOTHESIS AND AIMS

Endometriotic lesions engenders a unique inflammatory microenvironment capable of inducing kinase activity and surprisingly, a kinase-dependent lesion growth. AMPK/CYP4A/mTOR are cell signaling pathways that are activated by steroid hormones and growth factors leading to cellular events including gene expression, cell proliferation, migration, and survival. Hence, targeting AMPK/CYP4A/mTOR signaling axes may represent a potential novel treatment, and may also hold accountable for deep infiltrating endometriosis lesions. Nassif et al. have previously shown that ROS, NADPH oxidase as well as NOX1 and NOX4 are upregulated in endometriosis. For that, we aim to investigate the role of AMPK/CYP4A/mTOR signaling axes in inducing the proliferation of endometriotic implants. Therefore, we examined the expression of these pathways and sought to unravel whether their alteration exacerbates the disease.

Our hypothesis states that AMPK hypo-regulation, CYP4A alteration, mTOR hyperregulation, and elevated inflammatory cytokines induce endometrial cells proliferation and survival outside their niches. Moreover, AMPK hypo-regulation might play a pivotal role in CYP4A modulation. Consequently, leading to mTOR alteration and 20-HETE overproduction that induces fibrogenesis

CHAPTER 3

MATERIALS AND METHODS

3.1. Sample Collection

Institutional Review Board (IRB) approval was obtained for endometrial tissue from women who underwent laparoscopy at the American University of Beirut Medical Center. Patient consent forms were not required since the identity of the patient(s) remained utterly anonymous and knowing that research involving materials (data, documents, records, or specimens) collected for surgery or biopsy and intended to be discarded after pathology readings doesn't require informed consent. 15 consecutive anonymous deep infiltrating endometrial implants samples were collected from women suspected with endometriosis during the follicular phase of the cycle. Until inauguration of the study the collected samples were stored in liquid nitrogen. In order to reduce recurrence rates, it is preferable to excise normal tissue (margin) around the lesions. Thus, the excised implant would have a normal disease-free tissue. In this study normal tissues surrounding the implant were taken as control rather than eutopic endometrial tissues due to ethical and feasibility matters. All samples were histologically proven to be either endometriotic or normal.

3.2. Histological Analysis

Sections 5- μ m thickness from paraffin-embedded tissues were stained with Masson Trichrome and Hematoxylin and eosin (H&E). Hypertrophy and fibrosis were quantified using an image j analysis system.

3.3. RNA Extraction and cDNA Synthesis

Total RNA was isolated from the frozen endometriotic and control tissues by homogenization using a Dounce homogenizer in Trizol reagent, according to the manufacturer's protocol. Using NanoDrop ND1000 spectrophotometer, ratio of A260 to A280 was calculated to check for RNA purity. In order to generate cDNA, one microgram of the total RNA was taken using an Oligo (dT)18 primer, according to the protocol supplied with the Trizol kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit).

3.4. RT-PCR

In order to study gene expression of AMPK, mTORC1, RAPTOR, IL-6, IL-8, TNF- α , TGF- β , quantitative real-time PCR was performed with an iCycler 1Q Real-Time Detection System using the QuantiFast SYBR Green PCR Kit (Qiagen). The set of primers used for qPCR are as follows: AMPK (forward primer: CGTTCCTGTTCTGCTGGCT, reverse primer: TGTGACTGCCCAGGCGAGGT), mTORC1 (forward primer: CCCTGGTGGAGAGCCGGTGT, reverse primer: TCGGAATGCAGCCAAGCGGG), Raptor (forward primer: ACTGATGGAGTCCGAAATGC, reverse primer: TCATCCGATCCTTCATCCTC), GAPDH (forward primer: GTCAGTGGTGGACCTGACCT, reverse primer: TGACAAAGTGGTCGTTGAGG), IL-6 (forward primer: GCA CTG GCA GAA AAC AAC CT, reverse primer: CAG GGG TGG TTA TTG CAT CT), IL-8 (forward primer: ACT GAG AGT GAT TGA GAG TGG AC, reverse primer AAC CCT CTG CAC CCA GTT TTC), TNF- α (forward primer GCC CAT GTT GTA GCA AAC CC, reverse primer TAT CTC TCA GCT CCA CGC CA), and TGF- β

(forward primer : CAAGGGCTACCATGCCAACT, reverse primer AGGGCCAGGACCTTGCTG). The PCR reaction was carried out on 1 μ l of the produced cDNA with a total reaction mixture of 25 μ l. The PCR mixture was heat-denatured at 95°C for 10 min, followed by 45 cycles of 9 s at 95°C, 12 s at 61°C and 9 s at 72°C. Fluorescent signals were acquired at the last step of each cycle. A melting curve was calculated at the end of the cycles. The results were normalized to GAPDH, as reference genes, and to the normal control results (obtaining $\Delta\Delta Cq$). The fold change was calculated according to the formula: fold change = $2^{-\Delta\Delta Cq}$ (31).

3.5. Protein Extraction and Western Blot

Liquid nitrogen stored tissues were homogenized using tissue Dounce homogenizer in RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride. The homogenate was then placed on rotator overnight at 4°C. Afterwards the lysates were centrifuged at a maximum speed of 13200 rpm for 30 minutes. Finally, Protein concentration in the supernatants was measured using the Lowry Protein Assay.

For immunoblotting, 80 μ g of proteins were separated on 12% polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to polyvinylidene difluoride (PVDF) (Bio-Rad Laboratory, CA, USA). The blots were blocked with 5% BSA in Tris-buffered saline and then incubated overnight with rabbit polyclonal anti-CYP4A, (1:1000; Abcam) mouse polyclonal GAPDH (1:1000; Santa Cruz Biotechnology) was used as loading control. The primary antibodies were detected

using horseradish peroxidase-conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software (Youssef, Noureldein et al. 2021).

3.6. 20-HETE Production

Endometrial tissues were homogenized in a 10 mmol/l potassium phosphate buffer, pH 7.7, containing 250 mmol/l sucrose, 1 mmol/l EDTA, 10 mmol/l magnesium chloride, 2 μ mol/l leupeptin, 1 μ mol/l pepstatin, 2 μ g/ml aprotinin, and 0.1 μ mol/l PMSF. Microsomes were prepared by differential centrifugation as previously described (Nilakantan, Maenpaa et al. 2008, Wang, Tang et al. 2008) and used for 20-HETE measurement by high-performance liquid chromatography (HPLC). In short, [14 C]-labeled arachidonic acid (50–100 μ mol/l) was dried down and resuspended in the reaction mix containing 50 μ g microsomes, 30 mmol/l isocitrate, and 0.2-unit isocitrate dehydrogenase in reaction buffer (100 mmol/l potassium phosphate, pH 7.4, 5 mmol/l magnesium chloride, and 1 mmol/l EDTA). After incubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH to a final concentration of 1 mmol/l. Aliquots were removed at 30, 60, and 90 min, and the reaction was stopped by the addition of 100% methanol. The precipitated proteins were then pelleted by centrifugation (in a microcentrifuge), and the samples were stored at –20°C until analyzed. The metabolites were separated via HPLC on a C-18 column using an acetonitrile/H₂O gradient and identified by coelution with labeled standards.

3.7. Statistical Analysis

Results are expressed as means \pm standard errors (SE) and mean \pm SD. Two group comparisons were performed by Student's t-test. Statistical significance was determined as a probability (P value) of less than 0.05. All statistical analysis was performed with Prism 8 software (GraphPad Software).

CHAPTER 4

RESULTS

4.1. Endometriosis Morphology Structure

Endometriosis is characterized by endometrial glands and stroma outside the uterine endometrial lining with the ability to cycle and present the same cellular function as normal nascent endometrial tissue. However, this extra uterine endometrial tissue will follow subsequent cellular and histo-morphological changes throughout its regular development ending with subsequent fibrosis and atrophy. H&E stain was performed to assess morphology of the ectopic endometrial tissue in endometriosis. Our data presents a depiction of the different cellular and micro-morphological changes through the review of slides of confirmed cases with deep infiltrating endometriosis from peritoneal, endometrioma and vaginal nodule sections.

It is important to be aware of two major concepts as we analyze this pathological development. First, we must understand that the lesions do not present in a homogenous manner, meaning that different sections of the same lesion will be having different morphological changes because of the abnormal development of an already abnormally allocated tissue. Second, we must understand that this chronological depiction we are presenting is usually in an ideal scenario and does not necessarily apply to every non-nascent endometrial tissue.

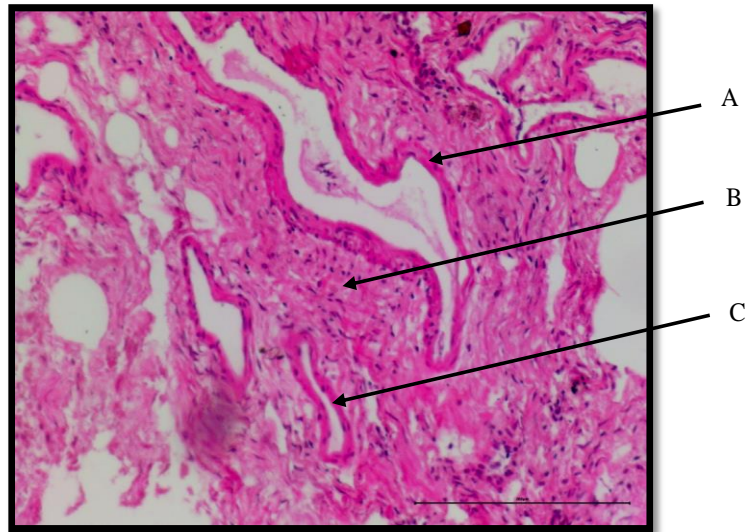


Figure 2. Nascent Endometrial Tissue with Few Dimorphic Features. (A) Appearance of the glandular apparatus: the presence of glandular epithelial tissue and the wide lumen. Few secretions are present within the lumen of the gland. (B) Presence of stromal cells in between the glandular structures and blood vessels. (C) Regular endometrial tissue is present with few and scarce signatures

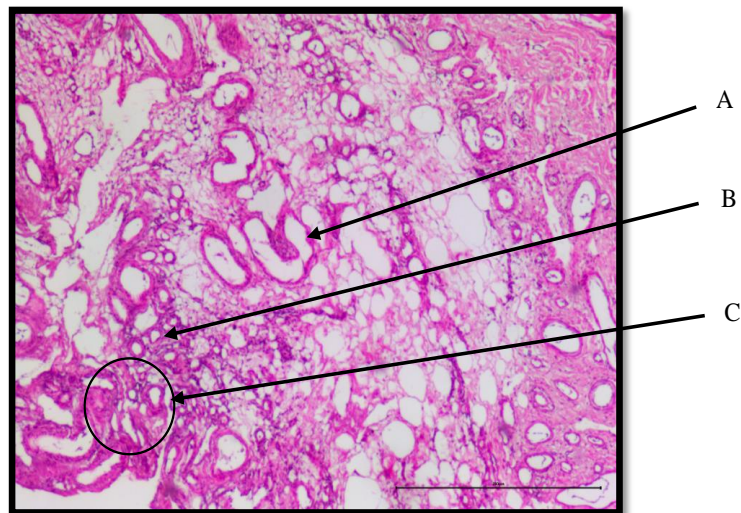
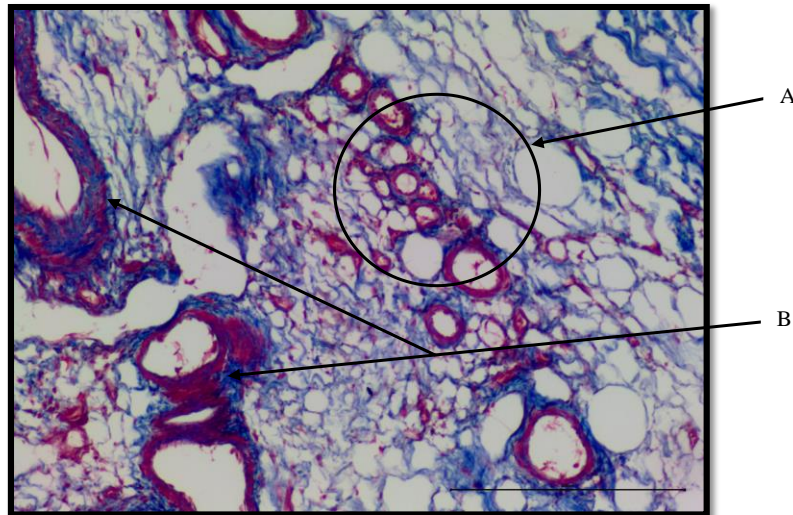


Figure 3. Early Hypervascularization of Endometrial Tissue (H&E). Normal morphology of reactive endometrial tissue, we encounter a high vascularity of the overall stroma with vessels of every kind. Observe that the overall stromal architecture is more or less intact and not yet necrotic. (A) presence of small and medium sized arteries. (B) presence of large arteriolar luminal entities. (C) large population of existing capillaries, inferred from the large metabolic demands of this rapidly

Highly vascularized section of non-nascent endometrial tissue. Notice the early deposition of collagen on stromal tissue accentuated under trichrome stain (blue). Figure



5. **Figure 4. Hyper vascularized Endometrial Tissue, (Trichrome).** (A) Different arterial and capillary morphologies juxtaposed on several areas. Notice the viability of the endothelial tissue accentuated by the trichrome stain (pink). (B) Glandular a venous structure and their viable

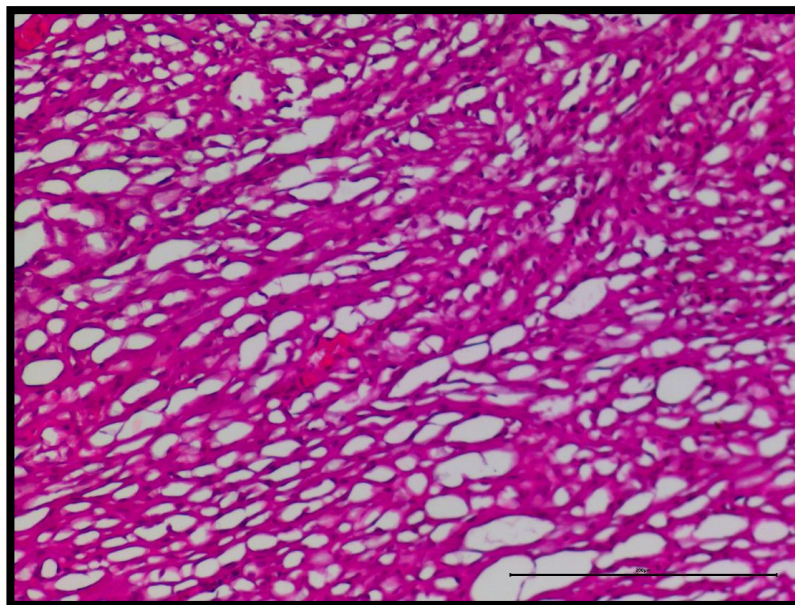


Figure 5. Hypercellular Endometrial Section (H&E). Hypercellularity of the stroma and the everlasting presence of minute disconnections in-between the stromal cells.

Once again, notice the hypercellularity of the stroma. Trichrome has accentuated the pattern of collagen deposition. However, notice the viable nuclei of the cells highlighted by a faint pink positivity. (Figure 7.)

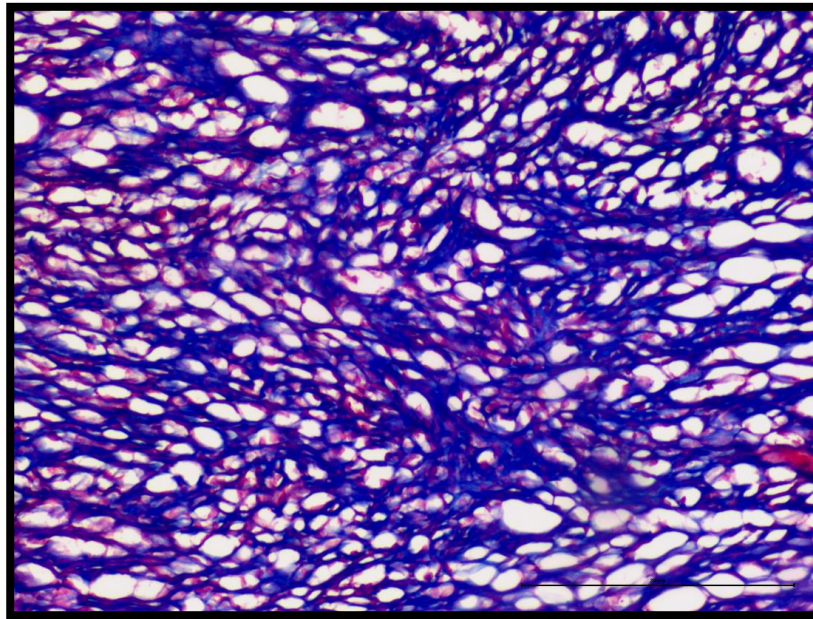


Figure 6. Hypercellular Endometrial Section (Trichrome).

Several factors contribute to the deposition of iron molecules within the non-nascent endometrial tissue. The high vascularity and damage of erythrocyte by the microenvironment are part of this pathologic picture. Iron deposits might be major culprits in the subsequent cellular damage, necrosis, and scar formation of the tissue.

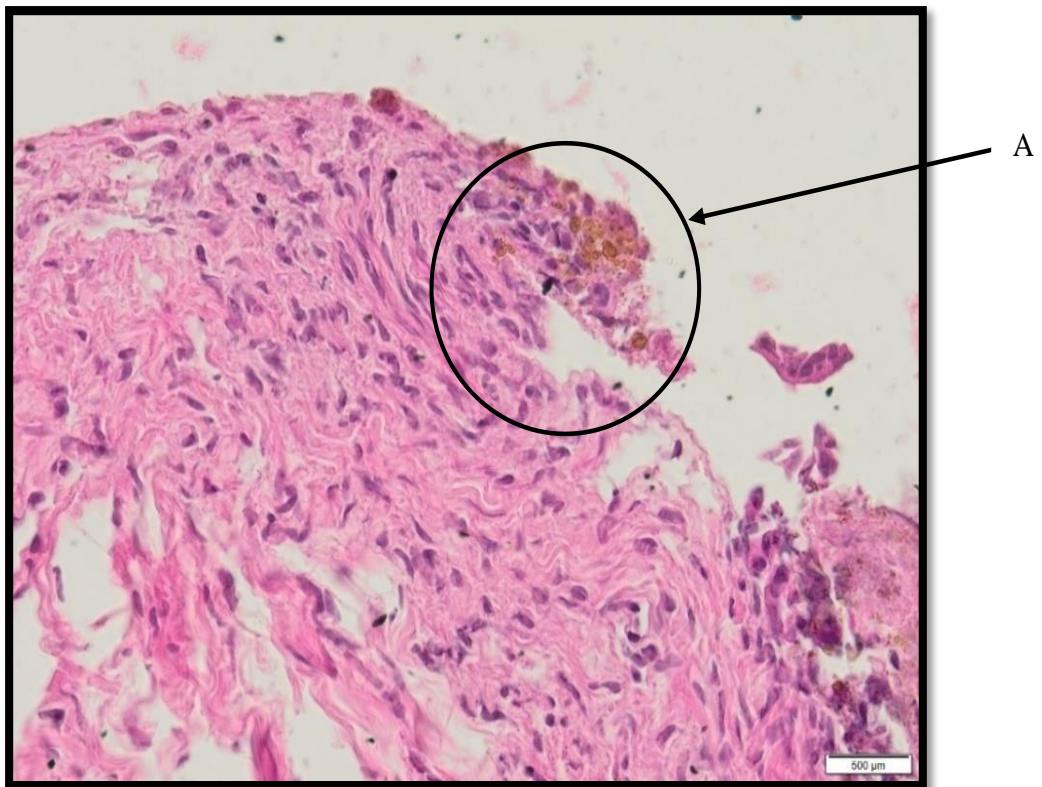


Figure 7. Iron Deposits (H&E). (A) iron deposits surrounding the stromal endothelial tissue.

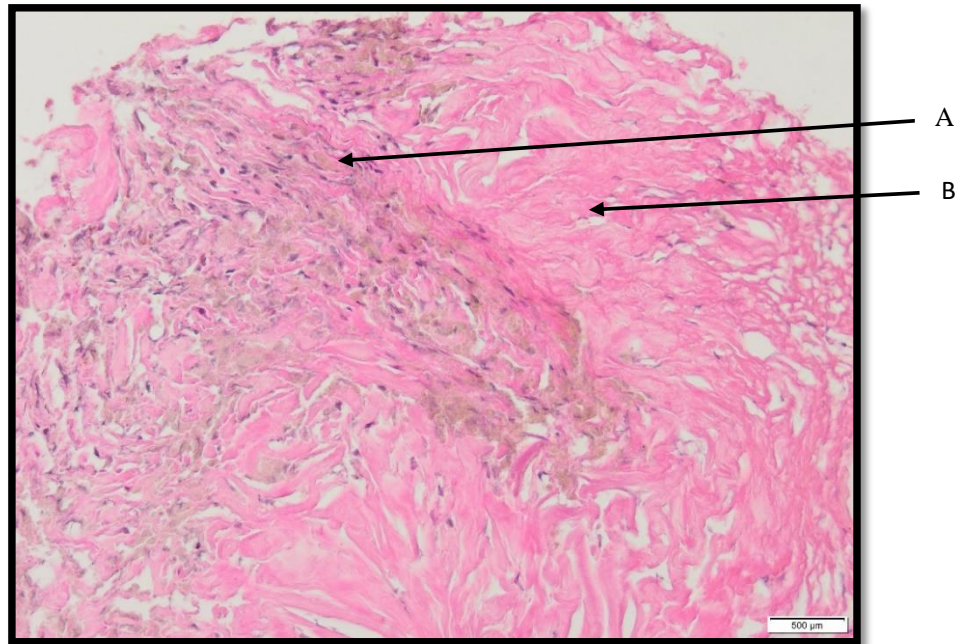


Figure 8. Dense Iron Deposits and Necrotic Tissue (H&E). (A) excessive accumulation of iron in viable stromal cells (denoted by their blue nuclei). (B) large anucleated area with amorphous cellular material characteristic of injured tissue and collagen deposition with subsequent scar formation.

This architecture demonstrates that the insult begins at the stromal end with viable glands remaining until the end fibrotic stages.

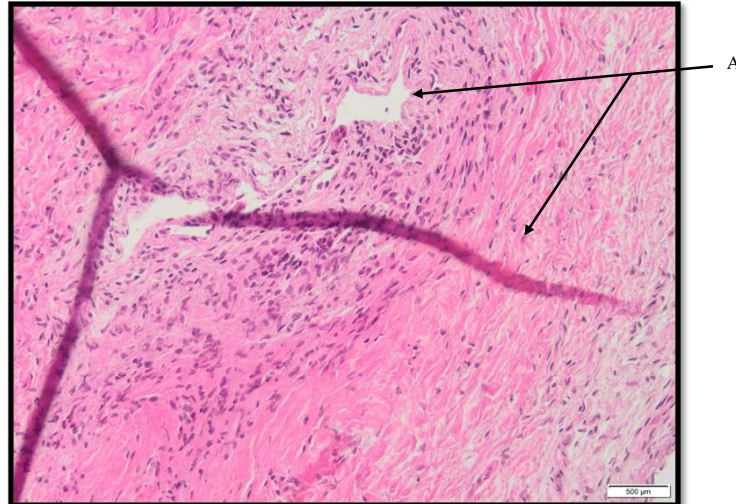


Figure 9. Remnants of Glandular Architecture (H&E). (A) Large glandular structure in the middle surrounded by a large rim of fibrous tissue.

End stages of with collagen deposition (blue) and development of a fibrotic scar. (Figure 11).

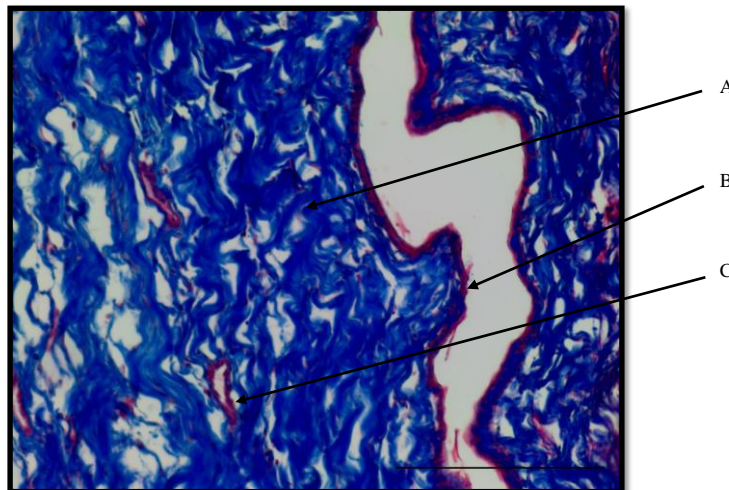


Figure 10. Remnants of Glandular Architecture (Trichrome). (A) anucleated pattern of the stroma and hyperdeposition of collagenous material. (B) weak positivity of the epithelial component of the gland, soon to be clogged by the collagen deposits. (C) presence of few non-viable blood vessels.

This final section depicts the overall histological changes that follow the probable chronology of an extra uterine endometrial tissue with many of the changes explained above. This really accentuates the heterogeneous nature of the lesion in terms of pathophysiological changes.

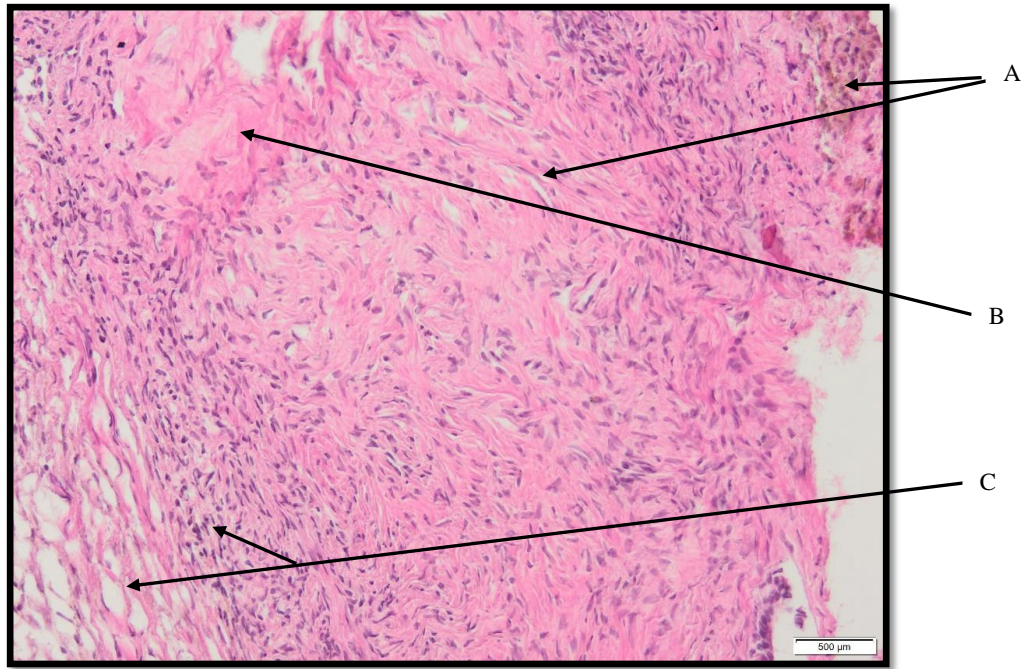


Figure 11. Heterogeneous Lesion (H&E). (A) Iron deposits with subsequent necrotic changes. (B) Amorphous material with collagen juxtapositions. (C) Hypercellularity.

4.2. Increased Collagen Deposition, Marker of Fibrosis

Fibrosis is a ubiquitous disease mechanism that can affect body organs singly or systemically. Scarring from fibrosis replaces parenchyma and/or connective tissues to adversely affect functional capability and this phenotype is prominent in endometriosis. Masson staining showed significant collagen deposition and hence degree of fibrosis in endometriosis compared to control.

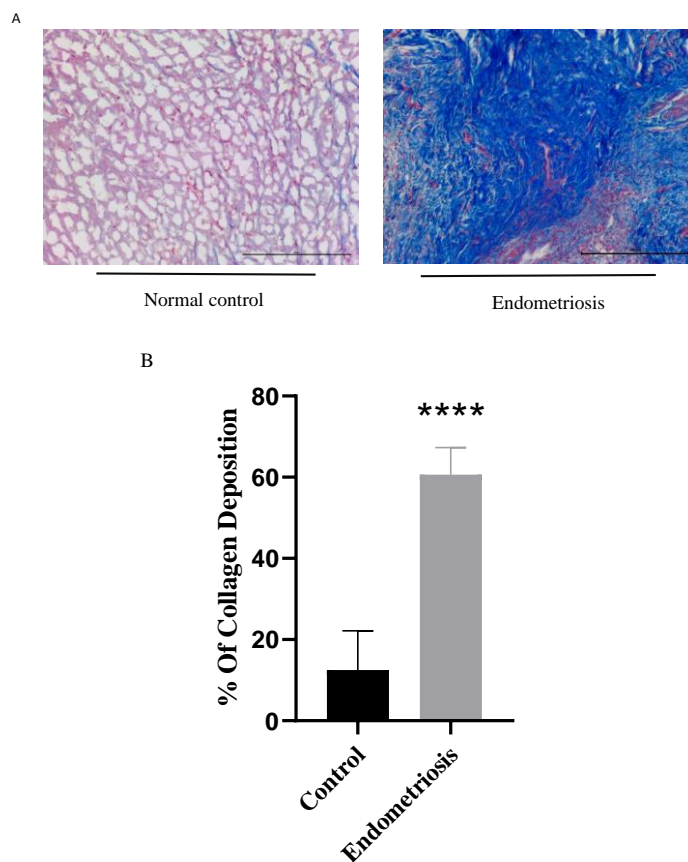


Figure 12. Percentage of Collagen Deposition in Women with Endometriosis Compared to Control. (A) representative image of masson trichrome stained normal tissue (left) and endometriosis (right). (B) Histograms showing % of collagen deposition (Blue color) using image j. Values are the means \pm SE unpaired t-test. *, $P < 0.05$ versus control.

4.3. Altered Expression of AMPK

AMPK is a well-established signaling pathway that plays a crucial role in cellular and redox homeostasis. Alteration in this pathway leads to increase reactive oxygen species production. Using RT-PCR, we assessed AMPK expression which was significantly decreased in women with endometriosis compared to control.

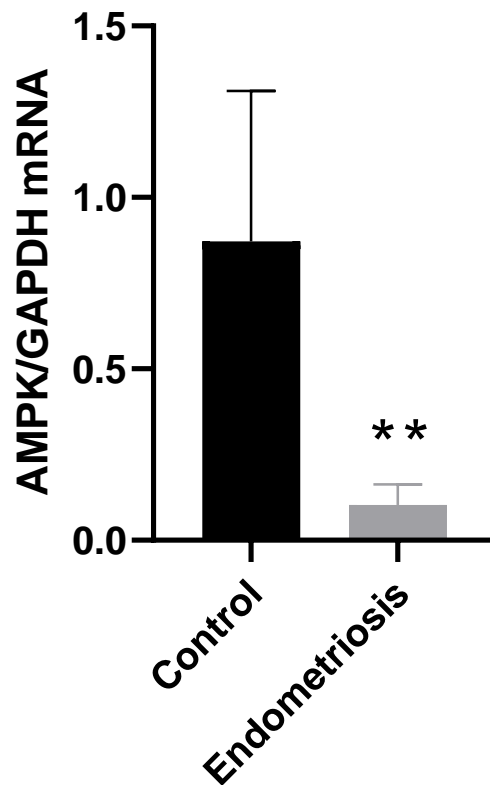


Figure 13. AMPK Expression in Endometriosis Compared to Control. Values are the means \pm SD t-test unpaired. *, $P < 0.05$ versus control

4.4. Upregulated CYP4A Induces 20-HETE Overproduction

CYP4A and its metabolites, 20-HETE, is well known in its role in inflammation and angiogenesis. Using western blot, we assessed CYP4A expression which was significantly increased in women with endometriosis compared to control.

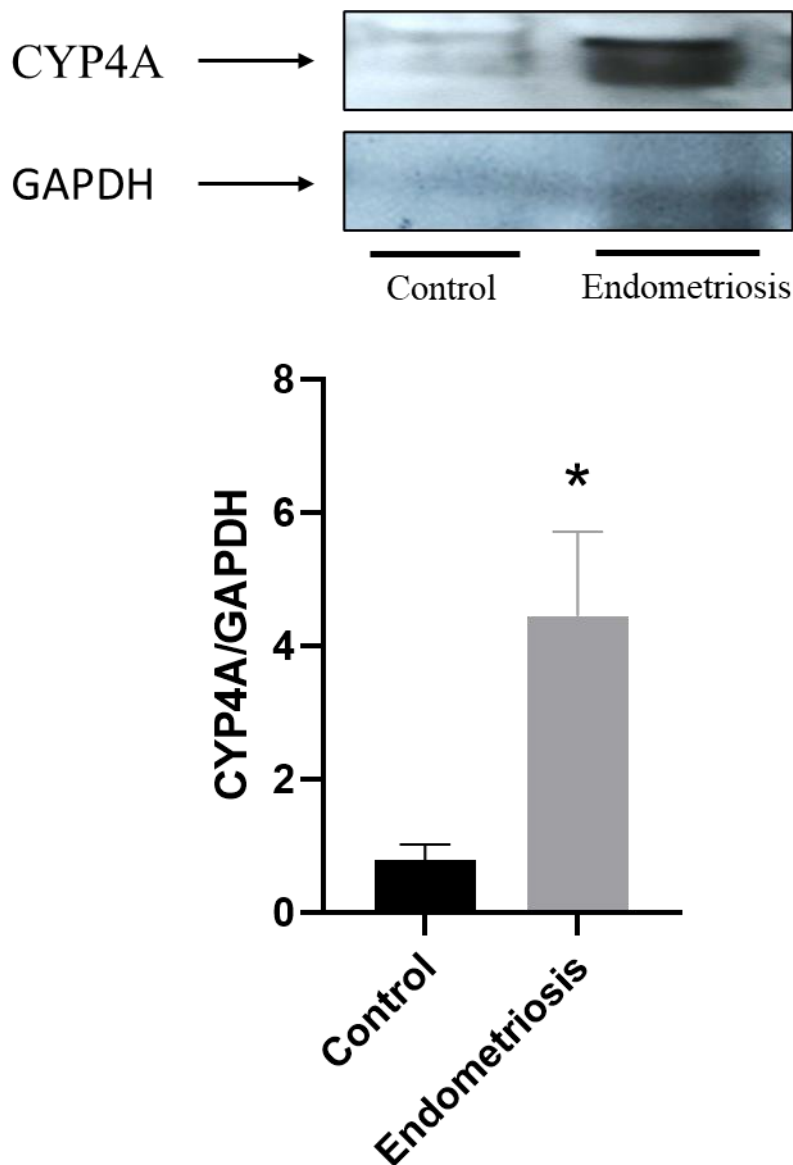


Figure 14. Assessment of CYP4A Expression. (A) Representative image of the expression of CYP4A. (B) Histogram showing increased expression of CYP4A in endometriosis compared to control. Values are the means \pm SE t-test unpaired. *, $P < 0.05$ versus control

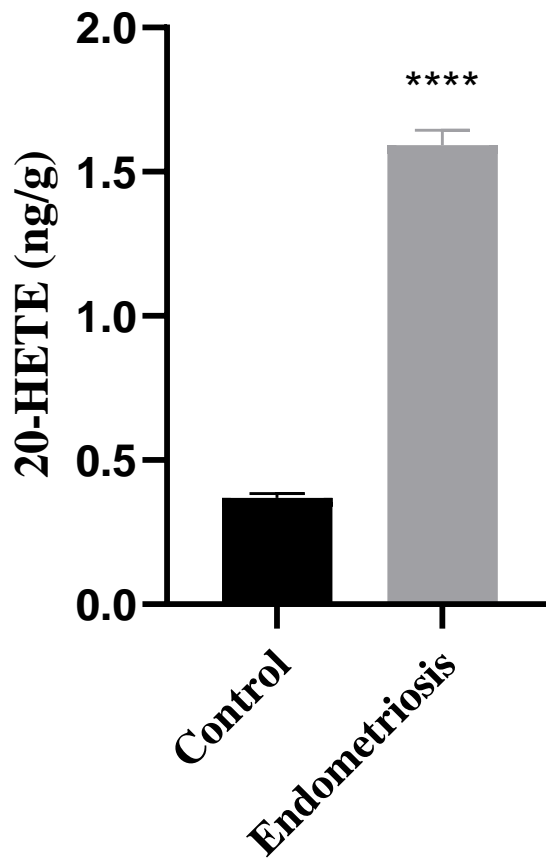


Figure 15. 20-HETE Levels in Endometriosis Compared to Control. Values are the means \pm SEM t-test unpaired. *, $P < 0.05$ versus control

4.5. Altered Expression of mTOR and mTORC1 specific subunit Raptor

mTOR is a central signaling regulator of cell metabolism, proliferation, differentiation, and survival. Raptor, mTORC1 specific subunit, has a positive role in nutrient-stimulated signaling to the downstream effector S6K1, maintenance of cell size, and mTOR protein expression. Using RT-PCR, we assessed mTOR and raptor expression which was increased in women with endometriosis compared to control.

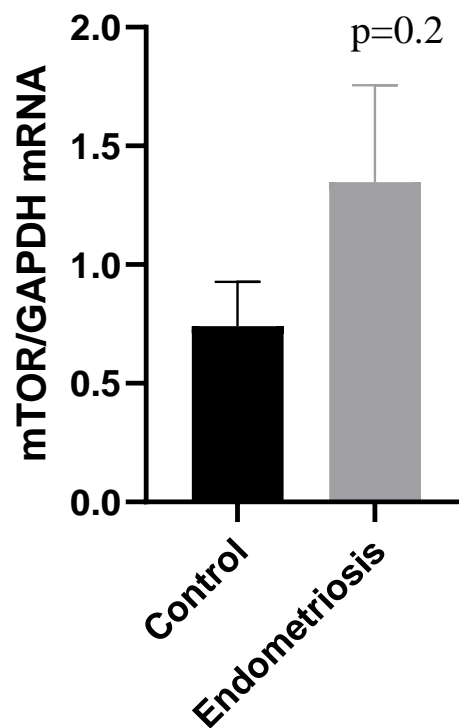


Figure 16. mTOR Expression in Endometriosis Compared to Control. Values are the means \pm SE t-test unpaired. *, $P < 0.05$ versus control

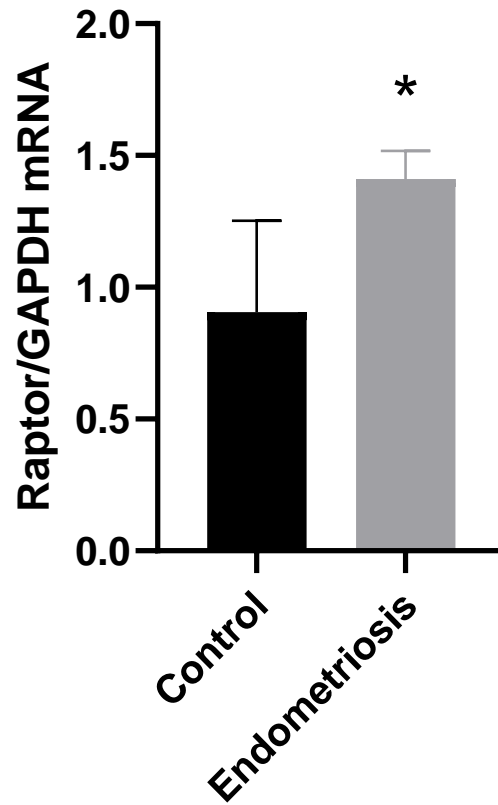


Figure 17. Raptor Expression in Endometriosis Compared to Control. Values are the means \pm SE t-test unpaired. *, $P < 0.05$ versus control

4.6. Increased Expression of TGF- β

TGF- β overexpression in various tissues is well-established to induce marked fibrotic changes. Using RT-PCR we assessed TGF- β expression which was significantly increased in women with endometriosis compared to control.

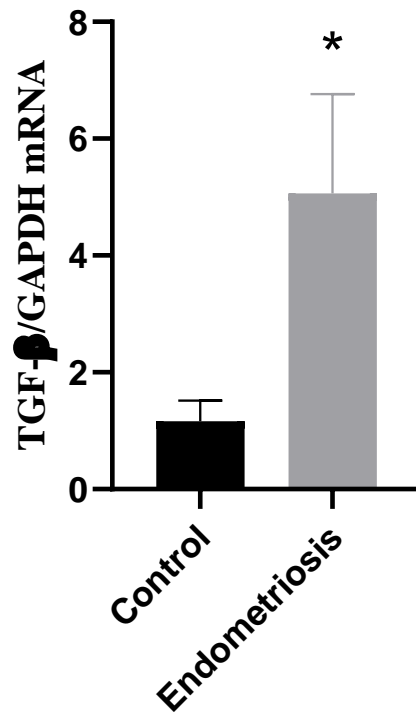


Figure 18. TGF- β Expression in Endometriosis Compared to Control. Values are the means \pm SE t-test unpaired. *, P < 0.05 versus control

4.7. Increased Expression of Inflammatory Cytokines

Inflammation is a well-recognized phenotype in endometriosis. Using RT-PCR we assessed TNF- α , IL-6 and IL-8 expressions. Inflammatory cytokines showed significant increase in endometriosis compared to control.

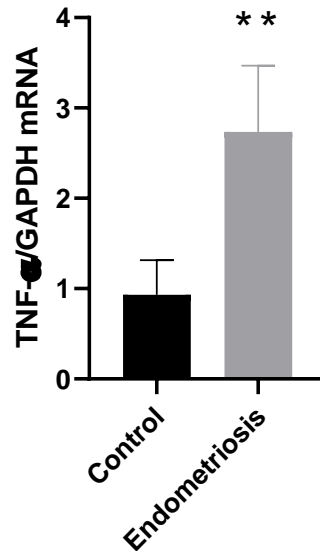


Figure 19. TNF- α Expression in Women with Endometriosis Compared to Control. Values are the means \pm SD t-test unpaired. *, P < 0.05 versus control

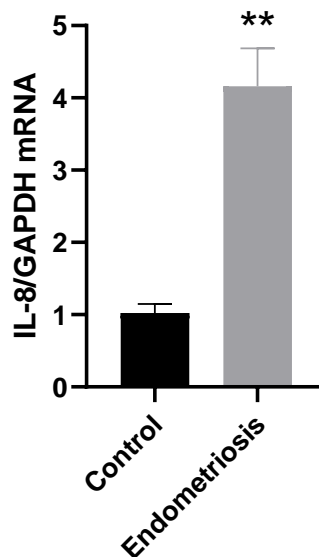


Figure 20. IL-8 Expression in Endometriosis Compared to Control. Values are the means \pm SE t-test unpaired. *, P < 0.05 versus control

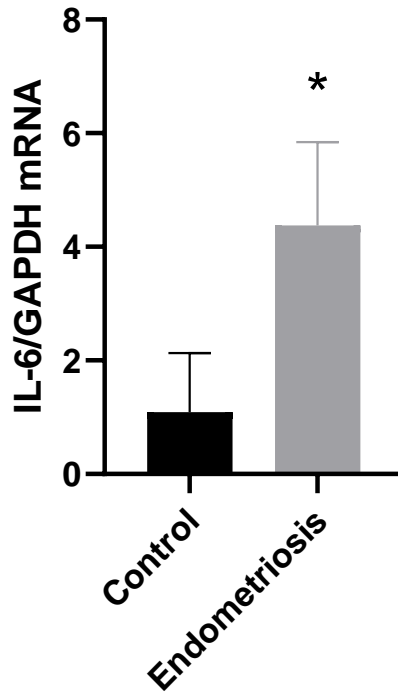


Figure 21. IL-6 Expression in Endometriosis Compared to Control. Values are the means \pm SD t-test unpaired. *, P < 0.05 versus control

CHAPTER 5

DISCUSSION

The role of CYP4A and its metabolite 20-HETE have been extensively studied in the pathogenesis of chronic diseases such as Alzheimer's disease, cardiovascular diseases, and diabetes, where 20-HETE is a common character (Eid, Maalouf et al. 2013). In this study, we demonstrated that endometriosis is associated with significant alteration in the expression of AMPK signaling pathway. This is accompanied with altered expression of CYP4A, its metabolites, 20-HETE and mTOR signaling pathway. Interestingly, modulation in AMPK/CYP4A/mTOR signaling axes was associated with increase in inflammatory cytokines, TGF- β and collagen deposition, markers of fibrosis.

When endometrial cells are outside the uterus, the immune system will be activated. Macrophages degrade erythrocytes and release proinflammatory cytokines for vasodilation and increase permeability of blood vessels, allowing extravasation of recruited leukocytes from blood vessels into the tissue to facilitate tissue repair. Besides, the degradation of erythrocytes allows the release of iron. Higher levels of ferritin, iron, and hemoglobin in the different components of the peritoneal cavity are observed in women with endometriosis compared to women without (Scutiero, Iannone et al. 2017). Iron homeostasis is important in the context that iron acts as a catalyst for the Fenton reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$. Hence highly toxic hydroxyl free radicals are produced and lead to overproduction of reactive oxygen species. This iron overload was distinctly visible in the histological samples using H&E stain. Defrere et al. reported that the injection of erythrocytes in murine model induced epithelial cells growth in endometriotic lesions. While the injection of desferrioxamine,

an iron chelator, inhibits this process. This postulates that iron may be responsible for endometriotic lesion growth (Defrère, Van Langendonck et al. 2006).

Downregulated AMPK is important in the context that this pathway is well-established energy sensor (Mihaylova and Shaw 2011). Thus, modulation in its expression leads to altered metabolism and imbalance redox homeostasis. It has been shown that altered AMPK leads to increased reactive oxygen species through NADPH oxidase-dependent mechanism (Eid, Ford et al. 2010). Nassif et al. showed that in endometriosis there is an increase in NOX1 and NOX4-ROS production (Nassif, Abbasi et al. 2016). In pertinent with these finding, our results have shown that decreased AMPK is accompanied with CYP4A alteration and 20-HETE overproduction, another source for ROS production.

Moreover, hyporegulation of AMPK could be responsible for the inflammation and infertility in women with endometriosis. Endometriosis is described as highly inflamed microenvironment with upregulation of IL-1, TNF- α and NF- κ B (Lin, Chen et al. 2018). This is well observed in our results as we have shown downregulated AMPK was accompanied with increased inflammatory markers such as IL-6, IL-8 and TNF- α . AMPK has been also shown to attenuates inflammatory pathway (Foretz, Guigas et al. 2014, Pan, Jiang et al. 2019). Several studies showed that AMPK acts as anti-inflammatory agent by inhibiting NF- κ B (Lee and Imm 2017). However, the mechanism is not well understood. It has been suggested that suppression of genes and activation of SIRT1, FOXO and PGC1 α genes are involved (Salminen, Hyttinen et al. 2011). Thus, the hypo regulation of AMPK accounts for the attenuation of anti-inflammatory pathway. Infertility is another problem facing women with endometriosis. About 30% of women with endometriosis are infertile. A recent study shows that

AMPK plays a functional role in female fertility (McCallum, Pru et al. 2018). After breeding of knockout AMPK subunit in female mice, litter sizes were reduced, and all female mice experienced premature reproductive senescence or dystocia. In addition, uterine histology showed endometritis, a condition with extensive endometrial fibrosis and disordered stromal-glandular architecture. These results suggest that AMPK essential role in female fertility (McCallum, Pru et al. 2018).

Increased CYP4A is held accountable for the unique microenvironment found in endometriosis. It is well known that CYP4A epoxygenase enzymes have been implicated in cancer progression by driving angiogenesis through VEGF activation (Yu, Chen et al. 2011). Interestingly, angiogenesis is extensively common in endometriotic implants. Studies have shown that angiogenesis occurs because of the higher concentration of VEGF in women with endometriosis compared to control (O, Fassbender et al. 2019). Furthermore, 20-HETE is a proinflammatory and stimulates NF κ B activation which is a main driver for inflammation and pain (Ishizuka, Cheng et al. 2008). Thus, CYP4A and 20-HETE could be the main cause for inflammation, Pain, and angiogenesis via VEGF activation. In addition, 20-HETE is a main source for reactive oxygen species production. Overproduction of ROS is responsible for the angiogenic factors in women with endometriosis (Ushio–Fukai 2007). Recent studies demonstrated that reactive oxygen species correlate with proliferative nature of endometrial cells in endometriosis as well as invasion phenotype (Ngô, Chéreau et al. 2009). Thus, 20-HETE induces further redox imbalance and might alters mTOR signaling pathway which was observed in our results.

In order to grow and divide, endometrial cells must increase production of proteins, lipids, and nucleotides while also suppressing catabolic pathways such as

autophagy. Here comes the role of the hyperactivated mTOR. As discussed earlier about the cross talk between mTOR and AMPK, nevertheless, other factors trigger mTOR signaling pathway in endometriosis. From our results this could be due to overproduction of ROS from 20-HETE metabolites. Besides, endometriotic implants secrete estrogen locally and this also increase phosphorylation of Deptor and activates mTORC1 via nongenomic pathway (Cuesta, Gritsenko et al. 2019). mTORC1 regulates glucose, iron free radicals and oxidative stress (McKinnon, Bertschi et al. 2014). There is disturbed regulation of GLUT1 and GLUT4 membrane transport in ectopic tissue of women with endometriosis (McKinnon, Bertschi et al. 2014). This could be due to the aberrant mTOR pathway. In addition, studies show that there is higher level of ferritin, Iron, and hemoglobin in the different components of the peritoneal cavity in women with endometriosis compared to women without (Scutiero, Iannone et al. 2017). Since mTOR regulates iron homeostasis, iron overload could be the result of dysregulated mTOR pathway (Bayeva, Khechaduri et al. 2012). Once mTORC1 is activated it activates myriads of downstream proteins. It activates S6K1 and 4E-BP1, leading to cell growth, proliferation, and invasion (Morita, Gravel et al. 2015). Moreover, mTOR activates HIF- α , master angiogenic switch, through its well-known vascular endothelial growth factor (VEGF) activation (Karar and Maity 2011). Additionally, it activates NF κ B pathway; hence, cytokines production and inflammation (Weichhart, Costantino et al. 2008). Moreover, hyper activation of mTOR could be held accountable for the microenvironment found in endometriosis. Eid S et al. showed that mTOR amplifies oxidative stress by upregulating Nox1 and Nox4 expression and activity in diabetic nephropathy (Eid, Boutary et al. 2016). Nassif J et al. showed elevated levels of NOX1/4 mRNA and protein expression as well as increased ROS levels in

endometriosis (Nassif, Abbasi et al. 2016). Thus, mTOR also induces oxidative stress. Several studies have provided a wealth of information about how the mTOR signaling pathway operates in innate immunity (Säemann, Haidinger et al. 2009, Weichhart, Hengstschläger et al. 2015). A central paradigm validates that mTOR is triggered by innate immune cells and integrates it to guide and shape the effector response (Weichhart, Hengstschläger et al. 2015). This occurs gradually by cellular metabolism to provide energy and building blocks necessary for the subsequent immune response of the cell (Weichhart, Hengstschläger et al. 2015). Besides, mTOR stimulates in a cell specific manner central transcription pathway, such as NF- κ B, STAT3, HIF1 α , PPAR γ pathways, cytokines and proinflammatory markers (Brugarolas, Vazquez et al. 2003, Guo, Li et al. 2013). In endometriosis there is constitutive expression of NF- κ B, TGF- β , TNF- α , VEGF, HIF- α , Interleukins (1 β ,6,8) and chemokines (Liu, Cao et al. 2012, Guo, Li et al. 2013). Since mTOR supports many innate immune functions, a fair activation pattern of the mTOR network is vital. This could contribute to the abnormal observations found in our results in women with endometriosis where mTORC1 is highly activated. For instance, mTOR inhibition decreases S6k and TGF- β -induced Smad2/3 phosphorylation (Han, Lin et al. 2018). Thus, mTOR increases levels of TGF- β . Then TGF- β activates via Smad3 signaling pathway, the formation of α -smooth muscle actin (SMA)-positive myofibroblasts. This increase is correlated with lesions development (Young, Ahmad et al. 2017). The role of mTOR in endometriosis has been studied in the context of decidualization and survival. Reports consistently demonstrate that decidualization is absconded in the eutopic and ectopic tissues and cells in endometriosis (Kim, Taylor et al. 2007). foxO1 is a transcription factor vital for the decidualization of endometrial stromal cells. In endometriosis foxO1 levels were

lowered and this might be due to hyperactivation of mTORC1 (Southgate, Neill et al. 2007). A study showed that foxO1 expression was increased upon treatment mTORC1 inhibitors, medroxyprogesterone acetate and dibutyryl cAMP (Yin, Pavone et al. 2012). Therefore, mTOR pathway is implicated in the decidualization process. Collectively, hyperactivated mTOR could be the driver for oxidative stress, abnormal immune response, cell proliferation, migration, angiogenesis, and fibrosis in women with endometriosis. All this cause chronic pain, inflammation and render endometrium unviable for implantation and pregnancy.

Based on our results endometriotic implants secrete TGF- β , and proinflammatory cytokines such as IL-6, and IL-8, and TNF- α . This could explain endometriosis associated infertility. For instance, endometriosis may impair sperm mobilization through macrophage-secreted IL-6 motility (Yoshida, Harada et al. 2004). Moreover, TNF- α can damage sperm DNA by inducing apoptosis and oxidative stress, and halts sperm–oocyte fusion (Mansour, Aziz et al. 2009). Also, TNF- α is implicated in angiogenesis and neurogenesis (Landskron, De la Fuente et al. 2014, Borsini, Zunszain et al. 2015). In addition, studies have shown integration of elevated IL-8 levels is associated in cancer adhesion and invasion (Kuai, Wang et al. 2012). TGF- β is a pleiotropic cytokine that plays an important role in angiogenesis, immunoregulation and cancer (Prud'homme 2007). The cells of the immune system produce the TGF- β which is a master regulator of the immune response. It is well known for its implication in promoting fibrosis (Prud'homme 2007). This cocktail of secretions promotes a proliferative and angiogenic environment that enhances endometriosis development and progression.

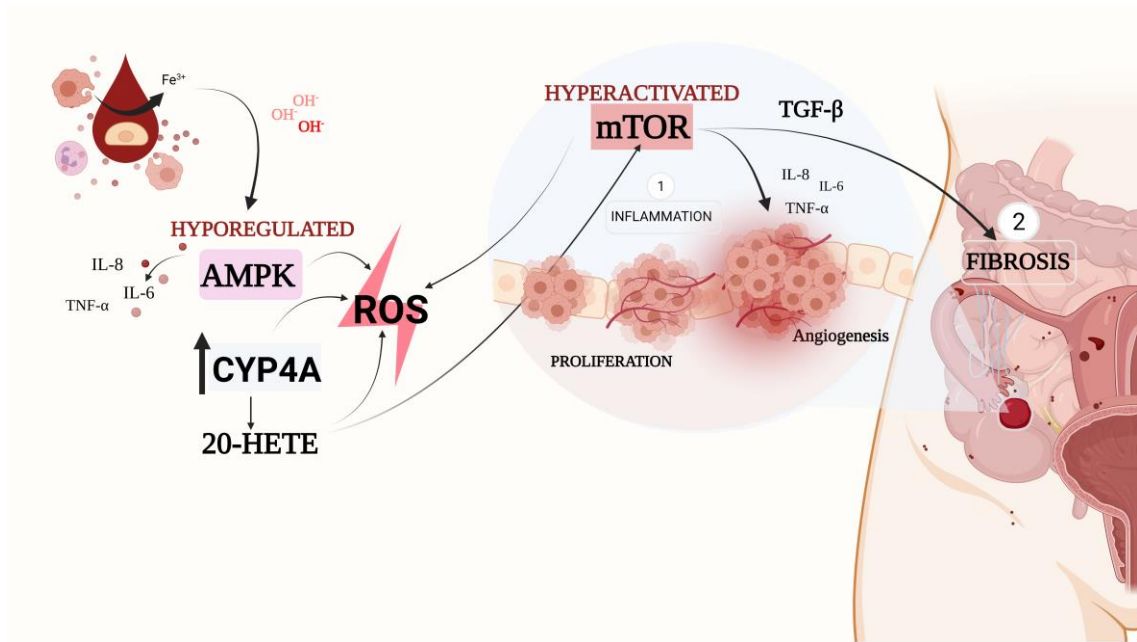


Figure 22. Role of AMPK/CYP4A/mTOR in the Pathogenesis of Endometriosis.
Assaf L. (Created using Biorender)

In summary, our data provides evidence for a novel function of the AMPK/CYP4A/mTOR signaling axes in the pathogenesis of endometriosis. Thus, AMPK activation, mTOR and/or CYP4A inhibition may represent a therapeutic modality of endometriosis.

One limitation of the study is the number of tissue samples. It is important to replicate our work on a large cohort of ectopic endometrial tissue. Also, further demonstration of the possible crosstalk between CYP4A and AMPK is required. Another limitation is that although mTOR inhibitors are already successfully used as therapeutic approach for cancer and to prevent the rejection of transplants. Studies are needed to translate experimental results to clinical use for female reproductive diseases. Nonetheless, mTOR is not the exclusive factor in female reproduction. mTOR orchestrates along with different factors in including folliculogenesis, ovulation, endometrium changes, or

embryonic development. Thus, the complete inhibition of mTOR would cause severe dose-limiting toxicities based on the indispensable role of mTOR in most human tissues.

Future Perspectives:

In order to strengthen our study further work should be accomplished

- ❖ Demonstrate cross talk between the two axes, CYP4A and AMPK.
- ❖ Demonstrate the main source of inflammation
- ❖ More drugs that target AMPK activation should be trialed, and studies should be performed
- ❖ Focus on the development of tissue-specific therapeutics (mTOR inhibitors) to avoid drawbacks associated with effects on unrelated tissues

CHAPTER 6

CONCLUSION

Endometriosis is a debilitating gynecological condition. It remains an enigmatic disease were its pathophysiology remains elusive. Current studies are addressing the role of genetic predisposition, epigenetic modulations, alteration of signaling pathways and oxidative stress. Despite the suggested theories endometriosis is a multifactorial disease and all disrupted factors are interconnected. In this study we addressed the hypo regulation of AMPK, alteration in CYP4A expression, 20-HETE overproduction, hyperregulation of mTOR, increased fibrosis and inflammation in endometriosis. Moreover, increased inflammatory cytokines and TGF- β were found in endometriosis. This latter is the main source for oxidative stress and consequently fibrosis. We also elucidated that mTOR contributes to proliferation and progression of the disease. Not only did we present new insights on the pathogenesis of endometriosis but also, demonstrated their manifestation in endometriosis associated symptoms, pain, and infertility. A deep understanding of the molecular basis of this disease can decipher the pathophysiology behind it. These observations may open the way to evaluate therapeutic approaches and diagnostic means. Hoping to better women's quality of life.

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