PLASMINOGEN ACTIVATOR INHIBITOR-1 IN ENDOMETRIOSIS

by

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Abstract

Endometriosis is a disease that affects almost 10% of reproductive-age women, where 50 % of these women have pelvic pain with sexual intercourse. Deep endometriosis is defined as an endometriotic lesion penetrating to a depth of 5 mm or more, and is characterized by both fibrosis (forming nodules) and invasion (into structures such as the colon). Other groups have found that increased plasminogen activator inhibitor-1 (PAI-1) or (*SERPINE1*) expression was associated with fibrosis and tumor invasion. In addition, a previous study found that the *SERPINE1* 4G allele (and thus increased gene expression) was associated with increased pain in women with endometriosis, and other work suggested that *SERPINE1* may be implicated in local neurogenesis.

The objective of this thesis is to determine whether PAI-1 expression is associated with a) deep infiltrating endometriosis; and b) deep dyspareunia. We propose that increased PAI-1 expression will be associated with deep infiltrating endometriosis, and increased pain in endometriosis via an increase in local nerve fibers. We utilized immunohistochemical analysis using a validated PAI-1 antibody. In the first cohort, we examined PAI-1 expression in deep infiltrating endometriosis and compared to endometrioma, superficial endometriosis, and eutopic endometrium. In the second cohort, we examined PAI-1 expression in cul-de-sac endometriosis from women with or without deep dyspareunia.

We found higher expression of PAI-1 in deep infiltrating endometriosis (n = 10) compared to superficial endometriosis (n = 10) ($p = 0.031$) and eutopic endometrium (n = 10) ($p = 0.002$). In

the second cohort, we found lower expression of PAI-1 in women with more severe deep dyspareunia ($r = -0.352$, $n = 35$, $p = 0.038$). However, there was no association between PAI-1 expression and local nerve bundle density.

In conclusion, we observed higher PAI-1 expression in deep infiltrating endometriosis, but lower PAI-1 expression in endometriosis from women with deep dyspareunia. Further research is needed to clarify the complexities of PAI-1 expression in endometriosis.

Preface

The research project presented in this thesis was originally developed in Drs. Bedaiwy and Yong laboratory, and covered by the Graduates Sponsorship Program at College of Medicine, Al-Imam Muhammad Ibn Saud Islamic University, Riyadh, KSA. The research was also funded by the Canadian Institutes of Health Research Operating Grants IHD-137431 and MOP-142273.

I participated in experimental design under the supervision of Dr. Yong and Dr. Bo Peng (post-doc), and conducted all listed experiments, data collection and analysis. A manuscript will be prepared for a future publication based on results from Chapters 4 and 5.

Publications

1- Nerve Bundles and Deep Dyspareunia in Endometriosis. Christina Williams, Lien Hoang, Ali Yosef, **Fahad Alotaibi**, Catherine Allaire, Lori Brotto, Ian S Fraser,,Mohamed Bedaiwy, Tony L Ng, Anna F Lee, Paul J Yong. Reprod Sci 2016 Jul 27;23(7):892-901.

2- Elevated Nerve Growth Factor and its Receptor Levels in Endometriotic Tissues are Associated with Deep Dyspareunia. Bo Peng, Hong Zhan, **Fahad Alotaibi,** Ghadeer Alkusayer, Mohamed A. Bedaiwy and Paul J. Yong (Submitted).

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List of Abbreviations

4G	Four sequential guanosines
5G	Five sequential guanosines
ASRM	American Society for Reproductive Medicine
BV	Blood vessels
COC	Combined oral contraceptive
COX-2	Cyclo-oxygenase-2
CPP	Chronic pelvic pain
DAB	Diaminobenzidine
DIE	Deep infiltrating endometriosis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptors
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GECs	Glandular epithelial cells
GREB1	Growth regulation by estrogen in breast cancer 1

GWAS	Genome-Wide Association Study
H-score	Histoscore
HepG2	Human liver hepatocellular carcinoma cell line
ICC	Immunocytochemistry
ID4	Inhibitor of DNA binding 4
IHC	Immunohistochemistry
IL1A	Interleukin 1A
KRAS	Kirsten rat sarcoma viral oncogene homologue
LCS6	Let-7 complementary binding site 6
MCP-1	Monocyte chemotactic protein 1
NB	Nerve bundles
NGF	Nerve growth factor
NSAIDs	Non-steroidal anti-inflammatory drugs
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor 1
PAI-2	Plasminogen activator inhibitor 2
PAI-3	Plasminogen activator inhibitor 3
PCI	Protein C inactivator

PCR	Polymerase chain reaction
PG	Prostaglandin
PGP9.5	Protein gene product 9.5
RCL	Reactive center loop
SCs	Stroma cells
SERPINE1	Serine protease inhibitor E1
siRNA	Small Interfering RNA
SMCs	Smooth muscle cells
SNPs	Single nucleotide polymorphisms
StAR	Steroidogenic acute regulatory protein
T-PA	Tissue-type plasminogen activator
TAFI	Thrombin-activatable fibrinolysis inhibitor
TGF-beta	Transforming growth factor-beta
TNFα	Tumor necrosis factorα
U-PA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEZT	Vezatin
VN	Vitronectin

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Finally, I would like to extend my deepest gratefulness to my parents and my wife, who have supported me throughout my years of education, both morally and financially.

Dedication

This is dedicated to the women and their families who are suffering from endometriosis.

Chapter 1: Introduction

1.1 Endometriosis

1.1.1 Definition and Epidemiology

Endometriosis is a common, estrogen-dependent, chronic gynecological disorder associated with pelvic pain and infertility. It is characterized by the presence of uterine endometrial tissue (glandular epithelium and stroma) outside of the normal location—mainly on the pelvic peritoneum, but also on the ovaries and other pelvic organs, and more rarely in the pericardium, pleura, and even the brain [1] [2] [3].

Endometriosis is a common debilitating disease that occurs in 6 to 10% of the general female population. The frequency of endometriosis has been found to be 35–50% in women with pain, infertility, or both [4]. The total annual cost of endometriosis in Canada is $1.8 billion [5].

1.1.2 Endometriosis Theories

1.1.2.1 Overview of Endometriosis Theories

There are three main theories of how endometriosis originates. The retrograde menstruation (Sampon's) theory is the most accepted theory of endometriosis pathogenesis [6] [7]. Meyer's theory of coelomic metaplasia and the lymphatic/hematologic spread theory are other known theories of endometriosis etiology. The theory of coelomic metaplasia suggests that endometrium-like tissue is differentiated from peritoneal mesothelial cells under sex hormone control, primarily estrogen. Lymphatic/hematologic spread theory suggests that viable endometrial cells travel from

the uterus through lymphatic or blood circulation to other body organs. These three theories of endometriosis will be reviewed here.

1.1.2.2 Sampson's Theory of Retrograde Menstruation

The origin of this theory is based on a paper entitled "Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation" [7], which was published in 1927 by John A. Sampson, M.D. The main focus of this paper was on retrograde menstruation and implantation as a mechanism by which endometriosis can develop. Sampson's observations during a number of hysterectomies performed during various stages of the menstrual cycle were the basis of this widely known theory. First of all, Sampson's theory suggests that the pathology of endometriosis occurs when live endometrial cells move retrograde into the pelvic cavity (refluxed through the fallopian tubes) instead of being shed from the uterus through the cervix into the vagina. These cells then implant themselves along various structures within and outside of the pelvic area and persist and grow there [7]. In the baboon, endometriosis can be induced by endocervical canal resection, which prevents endometrial cells from being shed from the uterus through the vagina, which simulates retrograde menstruation [8].

However, retrograde menstruation alone cannot account for the etiology of endometriosis, as retrograde menstruation occurs much more frequently than the prevalence of endometriosis in the population. Up to 90% of all women have retrograde menstruation, but only 10% develop endometriosis [6] [7]. It is clear that there are other factors besides endometrial tissue found outside of the uterus that are needed for the origin of this disease. This theory has led researchers to investigate other potential angiogenic, immunologic, and environmental factors that may cause the implantation of refluxed endometrial tissue outside of the uterus.

1.1.2.3 Meyer's Theory of Coelomic Metaplasia

The main focus of Meyer's theory or the theory of coelomic metaplasia is on ovarian and pelvic peritoneal endometriosis. This theory is supported by the fact that the epithelia of the ovary and the pelvic peritoneum, and that of the uterus, are derived from the same embryonal structure (coelomic-wall epithelium). It was suggested that the germinal epithelium of the ovary and the peritoneum of the pelvis can develop by metaplasia into endometrial tissue [9]. This theory does not depend on retrograde menstruation.

One argument against this theory is the fact that most metaplastic processes increase with advancing age, yet endometriosis is rarely found in menopausal women. Another argument is that if it is correct that peritoneal mesothelium cells can directly transform into endometrial–like cells, then endometriosis should be present in both women and men [6].

1.1.2.4 Lymphatic/Hematologic Spread Theory

Implantation of endometrial cells in ectopic locations may indicate the possibility of endometrial transit through the lymphatic or vascular systems. The observations of endometrial and endometriotic cells in lymphatic vessels and lymph nodes in some women have been explained by the lymphatic spread theory [3] [7]. Also, the presence of endometriosis in uncommon or distant sites such as the lungs may also be explained by lymphatic spread [11]. Some may argue that while this theory could potentially explain distant lesions it does not account for the more common pelvic location for endometrial lesions.

1.1.2.5 Genetics

It has been reported that endometriosis has a familial tendency. A family history of the disease is a factor in developing endometriosis [12], and there is some evidence that genetic factors may be involved in its pathogenesis [13] [14]. Many studies have focused on the identification of single nucleotide polymorphisms (SNPs) involved in endometriosis development [15].

Recently, several loci have been associated with the presence of endometriosis in Genome-Wide Association Studies (GWAS) [16]. These include the following: 1) rs13394619, an intronic splice variant in *GREB1* (growth regulation by estrogen in breast cancer. 2) rs1250248, located in an intron of *FN1*, which is a transcription factor-binding site. 3) rs7521902, an intergenic variant that is located 21 kb downstream of *WNT4* (The name "wnt" is a fusion of two terms, wg derived from the Drosophila gene wingless (wg) and int derived from the proto-oncogene integration-1). 4) rs7739264, an intergenic variant located 52 kb downstream of *ID4* (inhibitor of DNA binding 4). 5) rs1537377, and rs1333049, intergenic variants located 48 kb and 4 kb upstream of cyclin-dependent kinase inhibitor 2B antisense RNA (*CDKN2B-AS1*). 6) rs10859871, an intergenic variant, located in a regulatory region (DNAse hypersensitivity cluster present in 71/125 cell types), 17 kb upstream of vezatin (*VEZT*). 7) rs6542095, a downstream region variant located 2.3 kb of *IL1A* (interleukin 1A) [16].

Others have examined specific gene polymorphisms for an association with endometriosis. The main focus has been on genes regulating vascular function and tissue remodeling including

epidermal growth factor receptor (*EGFR*), vascular endothelial growth factor (*VEGF*), SERPINE1, and Kirsten rat sarcoma viral oncogene homologue (*KRAS*). Endometriosis is similar to cancer, as the implantation of endometrial cells ectopic locations requires neovascularization. Therefore, growth and other angiogenic factors, such as the *VEGF* and *EGFR*, could be linked to the pathogenesis of endometriosis.

The epidermal growth factor receptor (*EGFR*) is a growth factor receptor that induces cell differentiation and proliferation. The EGFR gene is located at 7p12. A study showed that a polymorphism in *EGFR*, *EGFR* +2073A/T SNP, is associated with high risk for endometriosis [17]. On the other hand, Inagaki et al found no significant differences in the frequency and genotype distribution of the *EGFR* +2073 A/T and *EGFR* +61 G/A polymorphisms between endometriosis patients with all disease stages and controls [18].

VEGF and its receptors are essential regulators of angiogenesis and vascular permeability, which contribute to the pathogenesis and development of endometriosis. The *VEGF* gene is located at 6p21.3 and consists of eight exons. Polymorphisms of the *VEGF* gene have been asspciated to the progression of endometriosis. In one of these studies, it was shown that T/T homozygotes and the T allele at the 5'-untranslated region of the *VEGF* gene are correlated with a higher risk of endometriosis [19]. Another study could not find an association between a specific polymorphism in the *VEGF* gene, +405 G/C polymorphism, and endometriosis [20]. This was conflicting with

the Bhanoori et al. study where it was shown that the +405 G/C genotype was higher in patients with endometrioma compared to controls [21].

Kirsten rat sarcoma viral oncogene homologue (*KRAS*) is a member of *RAS* gene family. The *RAS* family G proteins (N-, H-, and *KRAS*) are thought to play a critical role in the regulation of cellular proliferation. Targeting KRAS gene in the reproductive system of mice results in the development of endometriosis. This finding suggests that *KRAS* plays a major role in the progression of endometriosis [22]. A high frequency of *KRAS* 30 UTR variant rs61764370, which is located at microRNA (miRNA) let-7 complementary binding site 6 (LCS6), has recently been reported in endometriosis [23]. However, in a recent study, the variant was not associated with the development of endometriosis in Iranian women [24]. It was noted that higher expression of *KRAS* (mRNA levels) was observed in eutopic endometrium of patients with endometriosis compared to controls in the same study.

1.1.3 Staging of Endometriosis

According to the American Society for Reproductive Medicine (ASRM), endometriosis can be classified as stage I (minimal), II (mild), III (moderate), or IV (severe). This staging system depends on the number, the location, and the depth of implants and presence of adhesions. Examples of endometriosis stages are as follows: 1) Stage I (minimal): a few superficial implants 2) Stage II (mild): diffuse superficial implants and some adhesions. 3) Stage III (moderate): deep implants, endometriomas on one or both ovaries, and some adhesions. 4) Stage IV (severe): large

deep implants, large endometriomas on one or both ovaries, and many dense adhesions, sometimes with the rectum adhering to the back of the uterus (cul-de-sac obliteration).

1.1.4 Morphology

There are three anatomic sub-types of endometriosis: superficial peritoneal endometriosis, ovarian endometriosis cysts (endometriomas), and deep infiltrating endometriosis (DIE). Superficial endometriosis involves superficially attached implants, typically on the pelvic peritoneum. Ovarian endometriomas are cysts that contain thick chocolate-like fluid. These cysts (chocolate cysts) are often densely adherent to the peritoneum of the ovarian fossa. Deeply infiltrating endometriotic (DIE) nodules penetrate more than 5 mm beneath the peritoneum and may affect the vagina, uterosacral ligaments, bowel, bladder, or ureters [25]. The depth of infiltration is related to the type and severity of symptoms [26].

These anatomic sub-types can have different colors at surgery. The classic appearance is a "powder burn" lesions (blue or black). Also, endometriosis can appear as fibrosis (thick white tissue). Atypical or 'subtle' lesions are also common and include fluid-filled sacs (vesicles) and red or flame-like color.

1.1.5 Endometriosis in Different Locations

Endometriotic implants are seen in the pelvis and different sites in the human body. The most common location is the pelvic peritoneum and the ovaries. Endometriosis of the fallopian tubes is more rare [27] [28]. Lesions can also involve the urinary tract such as the bladder and ureters

[1] [30] [30]. In the gastrointestinal system, the most common site is the rectum and sigmoid colon. Much more rare examples are hepatic endometriosis, characterized by the presence of ectopic endometrium in the liver [31] [32].

The presence of endometriosis in sites outside the abdomen and pelvis is rare. Thoracic endometriosis syndrome is the presence of endometrial tissue in or around the lung causing catamenial pneumothorax [33]. A histologically confirmed case of endometriosis within the deep musculature of the thigh was reported by Gitelis et al. [34]. In the central nervous system, endometriosis is uncommon [35]. In a case report study, cerebral endometriosis was detected in a woman [36]. It was suggested that endometrial cells spread hematogenously to the brain through the vertebral venous system [37].

The malignant potential of endometriosis is rare (less than 1% of cases). The prevalence of ovarian cancer developing in women with endometriosis is higher than ovarian cancer in the general population, with specific associations with clear cell and endometrioid carcinomas [38]. Even more rare is malignancy in association with endometriosis of the rectovaginal septum, rectum, and sigmoid colon [39]. The risk factors for malignant transformation are not well understood. Prolonged exposure to unopposed estrogen hormone replacement following definitive surgery for endometriosis (hysterectomy and removal of both fallopian tubes and ovaries) may be a significant risk factor.

1.1.6 Symptoms and Treatments

1.1.6.1 Endometriosis Symptoms

Endometriosis can be associated with various symptoms. In particular, this disease is a common cause of infertility and pelvic pain, being found in approximately 50% of women with these symptoms [40]. There are four common types of pain related to endometriosis: dysmenorrhea, deep dyspareunia, dyschezia and non-menstrual chronic pelvic pain (CPP). Dysmenorrhea is defined as pelvic pain associated with menstrual bleeding [41], while dyschezia refers to painful bowel movements. In addition, deep dyspareunia (deep hitting pelvic pain with intercourse) is present in half of women with endometriosis [43] [44].

The devastating consequences of endometriosis-associated deep dyspareunia affecting women's health are well documented. Sexual activity and quality-of-life are reduced due to endometriosis-associated deep dyspareunia [44]. Although there is only a marginal correlation between stage and pain in general, there is an association between deep dyspareunia and deep infiltrating endometriosis of the cul-de-sac [45]. The cul-de-sac refers to the fold of peritoneum between the cervix/uterus and the rectum. Furthermore, endometriosis pain symptoms could be noncyclical, pain that has persisted for more than 3 months, or cyclical, dysmenothea (pain with menstruation), dyspareunia (pain with intercourse), dyschezia (pain with bowl movement), or dysuria (pain with urintation. In general, the pathophysiology of pain in endometriosis involves response to systemic estrogen levels (from ovulation), production of local estrogens, local inflammation and neuroangiogenesis, and central sensitization of the nervous system, which is sensation of that results from the activation of nociceptive pathways by peripheral stimuli (described below, section 1.1.7). In adult female rodents, widespread degeneration of sympathetic nerves in the

myometrium is observed when the levels of estrogen peak during proestrus and estrus [46]. This may indicate the role of estrogen in endoemtriosis pain.

An association between endometriosis and subfertility has also been described. This was shown on retrospective, cross-sectional and epidemiological studies in women and on nonhuman primate research [47]. Many mechanisms of how endometriosis significantly impacts fertility have been proposed. It was suggested that pelvic anatomy becomes distorted, and fertility is affected by mechanical factors such as pelvic adhesions. These adhesions prevent oocyte release or pick-up. Other mechanisms include altered sperm quality or function, decreased embryo quality, and disturbances in uterine contractility [48]. It has been reported that women with endometriosis exhibit an increase in apoptosis of the cumulus cells surrounding the oocyte [49]. Also, IL-6 was shown to cause a dose-dependent deterioration in microtubule and chromosomal alignment in the treated oocytes when compared to the untreated oocyte group [50]. Advanced stages of the endometriosis are more likely to occur in women who are infertile [47]. However, mechanisms by which mild disease impacts fertility is still not fully understood [51].

1.1.6.2 Endometriosis Treatment

Treatment of endometriosis often starts with analgesics such as acetaminophen/paracetamol and/or NSAIDs (non-steroidal anti-inflammatory drugs). Next line therapy is hormonal. The aim of hormonal treatment is to reduce the estrogen stimulation of the endometriotic lesions. Different types of hormonal treatment include: 1) Combined oral contraceptive (COC): which inhibit ovulation, reduce or stop menstrual bleeding, and directly inhibit endometriosis lesions. 2)

Progestins: similar mechanism as COC 3) GnRH-agonists: which induce a reversible menopausal-like condition.

If medical therapy is not effective, or if the patient desires pregnancy or has problems with side-effects, then surgical treatment is another option. Laparoscopy is the most common procedure used to surgically diagnose and treat endometriosis. Surgery can be used to relieve pain by removing the endometriosis, dividing adhesions or removing cysts. Definitive surgery is by hysterectomy and oophorectomy. Placebo-controlled randomized controlled trials have shown that surgical treatment of endometriosis improves pain and infertility [52].

1.1.7 Pathophysiology of Pain in Endometriosis

Endometriosis-associated pelvic pain is multifactorial. In some cases, pain is related to deep infiltrating endometriosis (DIE) invading visceral organs that are associated with adhesions and/or large bulk of disease (e.g. large ovarian endemetrioma cysts). DIE lesions have a specific anatomical distribution, as they are mainly found in the cul de sac and on the uterosacral ligaments. Therefore, it was hypothesized by some that this type of lesion is not caused by implantation of endometrial tissue during menstruation (i.e. Sampson's theory), but rather develops from metaplasia of Müllerian remnants located in the rectovaginal septum, in line with the in situ metaplasia theory [53]. Alternatively, there are those that believe that deep infiltrating endometriosis lesions develop from superficial peritoneal lesions in cul de sac [54].

However, most women with endometriosis do not have DIE, adhesions, or large cysts. Thus, there must be other mechanisms underlying pain. These can be divided into peripheral (inflammation and neurogenesis) and central (sensitization of the central nervous system). This thesis focuses on peripheral mechanisms.

1.1.7.1 Inflammation

Inflammation is one of the important mechanisms of how endometriosis can cause pain. There is increasing evidence that endometriosis is a pelvic inflammatory process, and endometriosis stimulates significant inflammatory responses. Proinflammatory cells such as macrophages, when infiltrated into endometriotic tissue, trigger release of proinflammatory cytokines and chemokines locally in the endometriotic tissue. The peritoneal fluid (PF) of women with endometriosis has been found to contain increased numbers of activated macrophages in many studies [55]. It has also been suggested that monocyte chemotactic protein 1 (MCP-1) as one of the major factors responsible for this increase of activated macrophages. Levels of monocyte chemotactic protein-1 in PF were found increased in moderate to severe endometriosis [56]. Macrophages synthesize and produce cyclo-oxygenase-2 (COX-2) and different cytokines into the peritoneal fluid including IL-1, IL-6, IL-8, IL-10, tumor necrosis factorα (TNFα), TGFβ, and VEGF [57] [58]. Prostaglandins, IL-1, IL-6 and TNFα and NGF, have also been found to be secreted directly from endometriotic lesions [59] [60] [61].

Moreover, mast cells have a major role in inflammatory reactions. They can release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines and

metabolites of arachidonic acid. In the setting of inflammation, mast cells are found close to primary nociceptive neurons and can release their inflammatory mediators such as NGF. Deeply infiltrating lesions show more mast cells near nerves than peritoneal and ovarian endometriosis [62]. Eventually, chronic inflammation develops in the surrounding tissue and is accompanied by a fibrous reaction, with the formation of local scarring and adhesions. The chronic inflammatory response to tissue injury involves both immune cell recruitment and mediator release during inflammation [63].

In addition, it was suggested that there might be a direct involvement of estrogen receptors in the inflammatory response in women with endometriosis. The correlation between cytokine production and ERa observed in women with endometriosis may increase the ratio of ERa/ERb in these patients and therefore reduce the anti-inflammatory action of ERb in the disease [64]. Prostaglandin E(2) is important as it increases estrogen synthesis by up regulating steroidogenic acute regulatory protein (StAR) and aromatase [59].

1.1.7.2 Neurogenesis

Local nerve fibers are thought to be involved in the mediation of pelvic pain and neuronal survival in women with peritoneal endometriosis, ovarian endometriosis, and deep infiltrating endometriosis [65]. Nerves have been suggested to follow vascular smooth muscle, endothelium, or their basal laminae (neuroangiogenesis) [66]. The presence of functional nerve fibers in endometriosis and corresponding uterine (“eutopic”) endometrium from women with endometriosis has been investigated by several researchers. The correlation between the density

and the severity of pain in endometriotic cases was examined as well. It has been hypothesized that endometriosis is associated with local neurogenesis, where an increased density of nerve fibres amplifies nociceptive signals to the central nervous system. For example, it was found that women with endometriosis have significantly higher nerve fiber density in comparison in peritoneal lesions compared to peritoneum from women without endometriosis [67]. This study, as have most studies in the field, utilized protein gene product 9.5 as a pan-neuronal marker. In the eutopic (uterine) endometrium, small nerve fibers were identified throughout the functional layers of the uterine endometrium in all endometriosis patients, but were not seen in the functional layer of the uterine endometrium in any of the women without endometriosis [68].

In deep infiltrating rectovaginal endometriosis, Anaf et al. specifically studied myelinated nerve fibers, stained with S100 [69]. The mean percentages of nerves located within the fibrosis of the nodule and within endometriotic lesions were significantly higher in group 1 (higher pain score) than in group 2 (lower pain score). Among nerves located within endometriotic lesions, there was a significantly higher proportion showing more intra- and peri-neural infiltration by endometriosis in group 1 than in group 2. It was also shown that there was a close histological relationship between nerves and endometriotic foci, and between nerves and the fibrotic component of the nodule. A high density of nerve fibers in deep infiltrating endometriosis (DIE), in particular in the uterosacral ligaments, were also demonstrated in two reports [71] [72].

Wang et al. demonstrated a significantly higher amount of nerve fibers in intestinal deep infiltrating endometriosis than in other deep infiltrating endometriotic lesions (e.g., cul-de-sac and

uterosacral ligament) [72]. In particular, the densities of nerve fibers stained with PGP9.5 and neurofilament were higher in rectal lesions than other sites. Nerve growth factor, tyrosine kinase receptor A, and p75 were strongly expressed in endometriotic lesions, and growth-associated protein-43 was also strongly expressed in the endometriosis-associated nerve fibers.

In recent work in our lab, immunohistochemistry for a pan-neuronal marker (PGP9.5) was performed in surgically excised endometriosis from the cul-de-sac in three clearly phenotyped groups [73]. The three groups of patients with cul-de-sac endometriosis were phenotyped as follows:

1) Study Group (n = 29) (tender endometriosis with deep dyspareunia): These patients had cul-de-sac endometriosis, and reported deep dyspareunia that was confirmed on endovaginal ultrasound-assisted palpation.

2) Control Group 1 (n = 17) (non-tender endometriosis with no deep dyspareunia): These patients had cul-de-sac endometriosis, and reported no deep dyspareunia and had no tenderness on endovaginal ultrasound-assisted palpation.

3) Control Group 2 (n = 12) (tender non-endometriosis with deep dyspareunia): These patients had cul-de-sac lesions that were not endometriosis (histologically normal or chronic inflammation), and reported deep dyspareunia that was confirmed on endovaginal ultrasound-assisted palpation.

For the primary analysis, there was a significant difference in PGP9.5 nerve bundle density between the three Groups (p = 0.003). Mean PGP9.5 nerve bundle density was significantly higher in the Study Group (1.16+/-0.56 bundles/HPF), compared to Control Group 1 (0.65+/-0.36, Tukey test, p = 0.005) and Control Group 2 (0.72+/-0.56, Tukey test, p = 0.044). Moreover, we found a correlation between higher nerve bundle density and more severe deep dyspareunia rated 0-10 (Spearman r=0.43, p=0.001). From these results, we concluded that a local increase in nerves (neurogenesis) may be a mechanism by which endometriosis causes deep dyspareunia.

1.2 The Plasminogen Activating System

My thesis is focused on the role of plasminogen activator inhibitor-1 (PAI-1), which is part of the plasminogen activating system, in the pathophysiology of endometriosis, in particular in DIE and in deep dyspareunia. The plasminogen activation system contains two plasminogen activators, tissue-type plasminogen activator (t-PA), and urokinase-type plasminogen activator (u-PA). These activators induce the conversion of plasminogen into the active plasmin. Several plasminogen activator inhibitors have been described, such as PAI-l, PAI-2, PAI-3 and TAFI (Figure 1.1). The interrelationship between the components u-PA, t-PA and PAI-1 will be reviewed here. Also, the involvement of these factors in the endometrium and endometriosis will be considered.

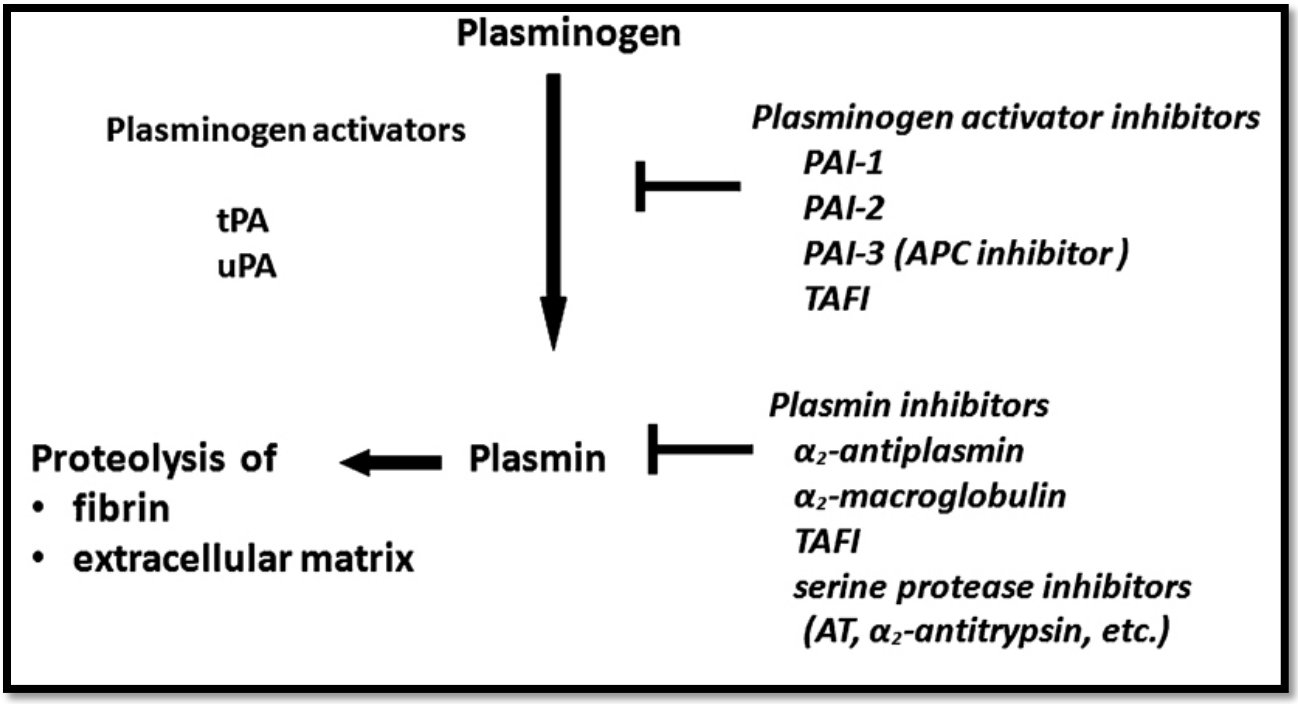

Figure 1.1: An illustration of the plasminogen–plasmin system.

tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; APC, activated protein C; TAFI, thrombin-activatable fibrinolysis inhibitor; AT, antithrombin [74].

1.2.1 Plasminogen Activator

The human gene u-PA is a 53-kDa serine protease produced as a single-chain protein [75], and t-PA is a 70-kDa protein which is secreted as a precursor in a single-chain form [76]. Both t-PA and u-PA can be converted to two-chain proteases upon cleavage by plasmin. The two-chain forms of plasminogen activators are held together by a single interchain disulfide bond.

Both single-chain and two-chain t-PA are enzymatically active. However, uPA is secreted as a single chain enzyme (pro-uPA) with very low intrinsic activity and is converted to active two-chain at the time of binding to the cell surface receptor uPAR. Structurally, both t-PA and uPA have the serine protease domain and kringle domains. t-PA has two kringle domains, and u-PA has only one. (Pro-)u-PA has a growth factor domain, important for binding to u-PAR [77].

t-PA and u-PA differ in their pathophysiological roles. t-PA primarily plays a role in fibrinolysis by binding to fibrin. t-PA was also found to contribute to adult central nervous system physiology [78] and is shown to play a role in plasticity and long-term memory [79]. In relation to their expression, t-PA has been reported to be expressed by oocytes [80] while u-PA is expressed by spermatocytes [81]. Both play roles in wound healing [82]. In tissue remodeling, u-PA is thought to play a more effective role than t-PA. These process include spermatogenesis [83], trophoblast invasion [80], wound healing [82], monocyte invasion [84] and angiogenesis [85], often together with u-PAR and PAI [86]. u-PA may also be considered as a key enzyme in the processes of cancer cell invasion and metastasis [87].

1.2.2 Plasminogen Activator Inhibitors

Uncontrolled secretion of tPA and uPA is potentially dangerous for cells. To protect themselves, cells secrete inhibitors to control pericellular proteolysis. Indeed, secreted uPA is often associated with PAI-1 and remains inactive [88].

Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors. The inhibitors include PAI-1, PAI-2, protease nexin 1, and protein C inactivator (PCI) [89] [90]. Serpins are relatively large molecules (about 330-500 amino acids), and they have a peptide bond for targeting proteases. The region responsible for interaction with target proteases, the reactive center loop (RCL), forms an extended, exposed conformation above the body of the serpin scaffold [91]. Serpin reactive center loop mobility is required for inhibitor function [92]. Generally, Serpins are present in most body fluids, tissues and cell lines [93].

Plasminogen activator inhibitor-1 (PAI-1), or *SERPINE1*, is a serine protease inhibitor (serpin) encoded by the human *SERPINE1* gene. PAI-1 is a single-chain 45- to 50-kDa glycoprotein secreted by many cell types. The mature protein consists of 379 amino acids and is encoded on chromosome 7. The gene spans approximately 12 kb and is composed of nine exons and eight introns. [94]. The reactive center loop of PAI-1 binds covalently to the active site serine of uPA or t-PA. Pro-uPA does not react with PAI-1, whereas two-chain active uPA rapidly associate with this inhibitor.

PAI-1 is a major inhibitor of fibrinolysis, a process that prevents blood clots from growing and becoming problematic. Increased PAI-1 activity results in depressed fibrinolytic activity. PAI-1 reduces fibrinolysis by inhibiting the conversion of plasminogen to plasmin through inhibiting t-PA and u-PA [95]. PAI-2 is a single-chain protein of 47 kDa. It is generally characterized as an inhibitor of the extracellular serine protease uPA. PAI-2 has the unique feature of its presence in two forms, secreted and cytosolic. In its secreted form, PAI-2 involved in control of tissue remodeling and fibrinolysis. In its cytosolic form, it contributes to intracellular proteolysis, and involved in processes such as apoptosis and inflammation [96] [97].

1.2.3 Plasminogen and Plasmin

Plasminogen binds to various proteins of the extracellular matrix, like fibronectin, laminin, type IV and collagen, and has the ability to degrade different matrix molecules.

Plasminogen receptors present on the cell surface of some cell types, including blood monocytes, granulocytes, and lymphocytes and endothelial cells. The two main plasminogen receptors are a-enolase and annexin II. Plasminogen occurs in blood plasma at the concentration of 12 μM and is also largely present in tissues. When plasminogen was targeted, mice were able to complete embryonic development, reach adulthood and reproduce. However, they were predisposed to severe thrombosis, and multiple spontaneous thrombotic lesions in liver, stomach, colon, rectum, lung, pancreas, and other tissues, as well as impaired skin wound healing [98].

Plasminogen is converted to the active enzyme plasmin by cleavage by plasminogen activators. Plasmin, a broad spectrum serine protease, degrades fibrin and extracellular matrix proteins. It

plays an important role in clot dissolution and extracellular remodeling. Plasminogen activators (PA, tissue-type or urokinase-type) mediate the conversion of the inactive proenzyme plasminogen to active plasmin by cleavage at a single arginine-valine bond.

1.2.4 Plasminogen Activator Inhibitor, Type 1(PAI-1) and Its Functions

Plasminogen activator inhibitor type 1 (PAI-1) is the principal inhibitor of the plasminogen activators responsible for the degradation of fibrin and extracellular matrix.

Although PAI-1 is one of the main regulators of the fibrinolytic system by binding uPA and tPA, it also has major effects on cell adhesion, detachment, and migration [99]. PAI-1 is a trace protein in plasma and has a short half-life in vivo. PAI-1 synthesis is highly balanced. These unique and diverse characteristics of PAI-1 may explain why PAI-1 expression correlates with a variety of diseases.

1.2.4.1 PAI-1 and Negative Regulation of Fibrinolysis

PAI-1 negatively regulates fibrinolysis. Because the urokinase-type (uPA) does not interact with fibrin, tissue-type plasminogen activator (t-PA) is the plasminogen activator involved in fibrinolysis. By inhibiting the activity of t-PA, PAI-1 negatively regulates fibrinolysis by inhibiting fibrinolysis. This regulation process occurs during clot formation and removal after injury [100]. When blood vessels are cut or damaged, the loss of blood from the system must be stopped. Fibrin, together with plasminogen, platelets, and other blood proteins, start to aggregate to initiate the clotting process. After bleeding stops, fibrinolysis occurs to remove fibrin clot. During fibrinolysis, t-PA is synthesized and released by endothelial cells. t-PA immediately surrounds the

clot to convert the bound plasminogen to plasmin. Plasmin then converts fibrin into fibrin degradation product [101]. The secretion of t-PA is very slow, and the fibrinolytic process takes days to be completed. This fibrinolysis process is tightly regulated by PAI-1 [95].

1.2.5 PAI-1 and the Human Uterine Endometrium

The human uterine endometrium is a unique tissue which proliferates rapidly after menstruation under the influence of estrogen and differentiates after ovulation under the influence of progesterone. The fibrinolytic activity in uterine fluid shows an increasing activity during the proliferative phase and then falls to its lowest levels in the secretory phase and then increases again after menstruation [102] [103]. Because PAI-1 is the main inhibitor of the PA system, its regulation is important to balance endometrial proteolysis. Koh et al. [104] found that t-PA activity and antigen concentrations were significantly higher in late secretory endometrium than in proliferative or early secretory endometrium. Also, it was reported that higher concentrations of PAI-1 antigen were seen in late secretory phase than in proliferative and early secretory endometrium. But, PAI-1 activity was not reported in the menstrual phase [104]. Casslen et al. have reported an increase in PAI-1 concentration after stimulating primary cultures of human endometrial stromal cells with progesterone [105].

1.2.5.1 PAI-1 and Endometriosis

PAI-1 not only inhibits the tissue/urokinase plasminogen activator (tPA/uPA), but also, PAI-1 is important in cell migration and invasion. The concentration of u-PA and PAI-1 were higher in uterine endometrium from women with endometriosis than in uterine endometrium from controls. Also, both u-PA and PAI-1 were even higher in endometriotic tissue than in uterine endometrium.

In this study, sample preparation included, endometriotic tissue samples, endometrial biopsy samples from healthy controls, and peritoneal fluid was obtained from women with endometriosis [106]. In a recent study, it was shown that endometriotic cells secreted more PAI-1 than uterine endometrial cells, and stromal cells (endometrial and endometriotic) secreted considerably more PAI-1 compared to epithelial cells [107]. Together, these results suggest that increased PAI-1 expression may be a feature of endometriosis.

1.2.6 PAI-1 and Human Pathophysiology

Both decreased PAI-1 levels and increased PAI-1 levels are associated with many human diseases. Therefore, the regulation of PAI-1 expression is very important. The reference range of PAI-1 is 2-15 AU/mL. The normal plasma concentration is 5-40 ng/mL [108]. Increased PAI-1 activity is observed in elderly individuals.

Increased PAI-1 levels are correlated with a variety of diseases including thrombosis, fibrosis, and invasive tumors [109]. In the cardiovascular system, PAI-1 overexpression leads to thrombosis by blocking fibrin degradation and vessel wall remodeling. PAI-1 levels can be used as an independent risk factor for cardiovascular diseases [109]. The elevated levels of PAI-1 in plasma are positively correlated with a variety of thrombotic vascular diseases, such as myocardial infarction [110], and deep venous thrombosis [109]. It has been previously reported that elevated levels of PAI-1 contribute to the development of venous but not arterial occlusions [111]. Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue in a reactive process. Elevated levels of PAI-1 was shown to associate with tissue fibrosis, and the overexpression is

found in some experimental fibrosis models [112]. Since PAI-1 is involve in the cardiovascular disease pathogenicity, it is important to know that the association between impairment in fibrinolytic activity and endometriosis was previously shown. For example, laparoscopically confirmed endometriosis was associated with increase women's risk for coronary heart disease (CHD) [113].

In addition, to promoting thrombosis/fibrosis, PAI-1 may be involved in invasion. Tumor invasion involves the breaking down of anatomical barriers and the migration of tumor cells into normal adjacent host tissues. These processes and tissue remodeling events are regulated by different proteolytic systems. The plasminogen activator (PA) system has been implicated in these processes. The idea that PAI-1 involved in cell invasion and migration was first suggested by Bajou et al [114]. Deficient PAI-1 expression in host mice prevented local invasion and tumor vascularization of transplanted malignant keratinocytes. When PAI-1 expression was obtained by intravenous injection of a replication-defective adenoviral vector expressing human PAI-1, invasion and associated angiogenesis were restored.

Moreover, Liu et al. investigated the importance of the urokinase (uPA)-plasmin system and PAI-1 in the human lung cancer cells invasion. Polyclonal antibodies, which inhibit uPA and PAI-1 activities, were utilized for the highly invasive H292 cell line. Invasive capacity was inhibited by antibodies to both uPA and PAI-1 in a dose-dependent manner. These data demonstrated that the uPA-plasmin system is important in promoting invasion into basement membranes. It seems that there should be a critical balance between uPA and PAI-1 for optimal invasiveness [115]. Utilizing

immunohistochemical staining, it was previously published that expression of PAI-1 was significantly higher in women with endometrial cancer stage III compared to stage I and II [116]. In this study, immunohistochemical expressions of uPA, PAI-1, and tPA did not differ between stage I and II patients. However, stage III subjects were found to have a significantly higher expression of uPA, PAI-1 and tPA. In addition, the patients who survived were found to be PAI-1 negative, while study participants with an unfavourable disease course were PAI-1 positive. In relation to angiogenesis, it was previously demonstrated that PAI-1 is coexpressed with the angiogenesis marker alpha(v)beta3 integrin in blood vessels of primary neuroblastoma tumors, suggesting that PAI-1 plays a role in angiogenesis [117].

PAI-1 is involved in tumorigenesis and metastasis by influencing pericellular proteolysis and cell migration during angiogenesis [115]. PAI-1 levels are elevated in many cancers including breast cancer [118], endometrial cancer [119] and lung cancer [120]. PAI-1 expressed by stromal fibroblasts and endothelial cells promotes tumor growth and spread. PAI-1 expressed by these cells therefore, seems to be a potential therapeutic target in cancer [121]. Although PAI-1 is a useful prognostic factor for the course of disease in early breast cancer, it may also serve as a predictive factor predicting response to systemic therapy [122]. On the other hand, there are some conflicting reports that PAI-1 may not be involved in invasion/migration in other contexts. For example, PAI-1 was found to block SMC (smooth muscle cell) migration [123] even though its stabilizer vitronectin, which is a major adhesive glycoprotein in blood, was reported to significantly enhance the migration of SMC [123]. In human ovarian cancer cell line, PAI-1 was found to reduce cell invasion by an in vitro invasion assay [124].

PAI-1 may be associated with local neurogenesis. PAI-1 is expressed in various cell types (as mentioned before). One of these cells are astrocytes [125]. Astrocytes release PAI-1 to enhance neuron survival not only by activating the MAPK/ERK pathway but also by activating the c-Jun/ AP-1 pathway [126]. PAI-1 produced by astrocytes protected neurons against N-methyl-D-aspartate receptor-mediated excitotoxicity [127]. PAI-1 allow the persistence of the local fibrin matrix that could act as a scaffold for nerve fiber formation [128]. Alternatively, there is some empirical evidence that increased PAI-1 expression helps to promote neuron survival, for example through a reduction in apoptosis [126]. Taken together, these findings suggest that PAI-1 has an important role in thrombosis/fibrosis, invasion/proliferation, and nerve growth.

1.2.7 Regulation and Genetics of PAI-1 Expression

PAI-1 gene expression is regulated by many factors including cytokines, growth factors, hormones, glucocorticoids, angiotensin II and fatty acids [129]. PAI-1 expression depends primarily on the cell type rather than the promoter context. For example, it was shown that Thymosin beta 4's effects on PAI-1 regulation were observed in endothelial cells but not in human fibroblasts [130]. However, PAI-1 regulation by glucocorticoids, transforming growth factor-beta (TGF-beta) and the phorbol ester PMA was shown to be exerted at the promoter level [131]. Another finding suggests the existence of a cell shape-dependent aspect PAI-1 expression control distinct from both the constitutive and growth factor-mediated pathways of gene regulation [132]. PAI-1 is expressed in many tissues including liver, lung, heart, kidney [133], blood platelets [134] and adipose tissue [135]. Also, it has been found to be expressed in the endothelium of blood vessels, nerve bundles, smooth muscle cells, and macrophages [136] [137] [138] [139].

The PAI-1 gene, or *SERPINE1*, is located at 7q21.3-q22 and consists of 9 exons. An insertion/deletion polymorphism in the promoter region of the PAI-1 gene at position -675, named 4G/5G, results in either an allele with four sequential guanosines (4G), or five sequential guanosines (5G), and the 4G allele is associated with increased expression of PAI-1 [140].

1.2.8 PAI-1 Genetics and Endometriosis

Several studies have investigated the correlation between the PAI-1 polymorphism, 4G/5G SNP, and endometriosis development. A previous study found that the 4G allele frequency was significantly higher in those with endometriosis [141]. Also, in a recent meta-analysis study, Luyang et al. found a significant association between the PAI-1 4G polymorphism and increased risk of endometriosis [142]. Patients with the homozygote 4G/4G had a six-fold risk for endometriosis development compared with the homozygote 5G/5G. Also, it was found that for the 4G polymorphism, Asians and Brazilians had significantly increased risks for endometriosis compared to Caucasians. External or environmental factors may contribute to these differences as these factors may affect gene transcription activity. Nonetheless, these observations were not found in two other studies [143] [144]. In a study published in 2008, it was shown that the genotype and allele frequencies of PAI-1 4G/5G polymorphism did not differ significantly between patients with endometriosis and controls [143]. Also, Gentilini et al. could not associate the PAI-1 4G/5G polymorphism to high risk for endometriosis [144]. These conflicting findings could be explained by the fact that larger sample size provide more power to detect significant differences. For example, Luyang et al. meta-analysis included five studies with 644 cases and 777 controls, which is larger sample size compared to other studies (total 389 cases in Ramon et al. paper [143] and 368 cases vs 329 control in Gentilini et al. paper [144]).

1.2.9 PAI-I, Deep Infiltrating Endometriosis, and Endometriosis Pain/Neurogenesis

Given that increased PAI-1 expression has been associated with fibrosis and tumor invasion, it is plausible that PAI-1 expression would be increased in deep infiltrating endometriosis. DIE is characterized by both fibrosis (forming nodules) and by invasion (into structures such as the colon). Thus, I propose that PAI-1 expression may be increased in DIE.

In addition, in a sub-analysis, a previous study found that the PAI-1 4G allele (and thus increased PAI-1 expression) was associated with increased pain in women with endometriosis [141]. Moreover, as summarized above, PAI-1 may be implicated in local neurogenesis. Thus, I also propose that increased PAI-1 expression will be associated with increased pain in endometriosis, via an increase in local nerve fibers.

1.3 Objective

My objective is to characterize the expression of PAI-1, possibly related to the 4G/5G polymorphism, in deep infiltrating endometriosis and in endometriosis deep dyspareunia.

1.4 Aims

Aim 1: To determine the feasibility of genotyping the PAI-1 4G allele in formalin-fixed paraffin-embedded (FFPE) endometriosis tissues.

Aim 2: To validate an antibody for immunohistochemistry of PAI-1 and to use it to investigate the expression of PAI-1 in different types of endometriosis: a) deep infiltrating endometriosis b)

ovarian endometrioma; c) superficial endometriosis, in addition to a control group d) uterine (eutopic) endometrium.

Aim 3: To investigate the expression of PAI-1 in a pain study group (cul-de-sac endometriosis in women with deep dyspareunia) and a control group (cul-de-sac endometriosis in women without deep dyspareunia).

1.5 Hypothesis

PAI-1 4G is associated with increased expression, and increased PAI-1 expression is associated with DIE (via fibrosis/invasion) and with deep dyspareunia pain (via neurogenesis) (Figure 1.2).

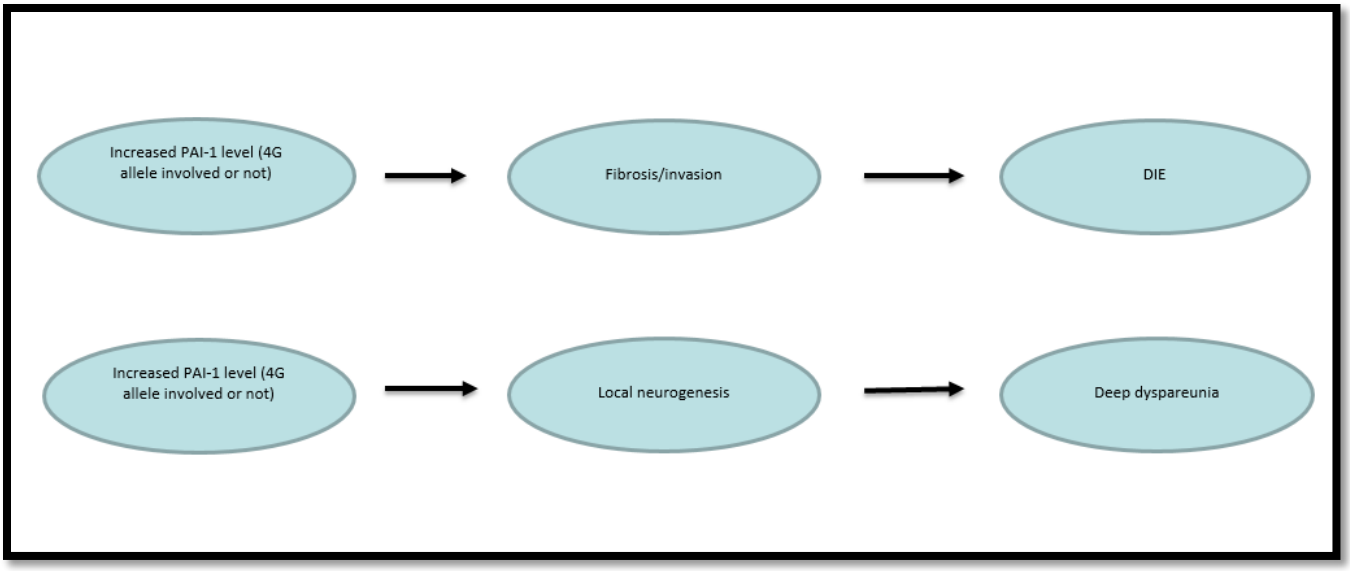

Figure 1.2: An illustration of my hypothesis.

PAI-1 4G is associated with increased expression, and increased PAI-1 expression is associated with DIE (via fibrosis/invasion) and with deep dyspareunia pain (via neurogenesis).

Chapter 2: Materials and Methods

2.1 Setting and Patient Groups

This study was carried out at an academic hospital-based program, the Centre for Pelvic Pain and Endometriosis, which is the tertiary referral center for British Columbia. All endometriosis tissues were obtained women who underwent laparoscopy to diagnose and/or treat endometriosis. The cases were retrospectively obtained from pathology archives at Vancouver General Hospital and BC Women's Hospital, from 2010-2013. Formalin-fixed, paraffin-embedded (FFPE) blocks were used for both deoxyribonucleic acid (DNA) extraction and immunohistochemistry. All the hematoxylin and eosin stained sections were histologically reviewed by a pathologist to identify samples with endometriosis glandular epithelium and stroma cells. UBC Ethics approvals: H11-02882 and H11-00536.

For Aim 1 and Aim 3, we used the Study Group and Control Group 1 from our previous study [73]. Again, the Study Group (n = 29) consists of tender cul-de-sac endometriosis in women with deep dyspareunia, while the Control Group (n = 17) consists of non-tender cul-de-sac endometriosis in women with no deep dyspareunia.

For Aim 2, we used a separate independent cohort, of deep infiltrating endometriosis (n = 10), ovarian endometriomas (n = 10), superficial peritoneal endometriosis (n = 10), and uterine (eutopic) endometrium (n = 10).

2.2 DNA Extraction from FFPE Blocks

We initially attempted to isolate genomic DNA from the FFPE blocks using a commercial kit (RecoverAll DNA Isolation Kit. Grand Island. USA). These attempts were not successful. We suspect that DNA does not bind to the membrane. Instead, it ends up in the flow through. Thus we prepared genomic DNA from FFPE blocks of the endometriosis patients using a phenol-chloroform (PC) extraction method (with assistance from Dr. Wendy Robinson's lab) as described below.

The PC extraction is a liquid-liquid extraction used for the isolation of DNA, RNA, and protein [145]. The FFPE tissue sections, 20 µm each, were deparaffinised in autoclaved centrifuge tubes by two changes of xylene, 1000 µl for 10 minutes at 60 °C. After spinning the tubes at 13,000 rpm for 5 minutes, the supernatant was discarded then alcohol rehydration series was performed by 100% and 95% ethanol rinses. After that, the tissue pellets were dried at 37 °C for 60 minutes. All pellets were digested with 20 µl proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and 300 µl digestion buffer (50 mM Tris-HCl, pH 8.5. 1 mM EDTA, 200 µl of 10% Tween 20). All samples were incubated at 55°C in a water bath overnight. Proteinase K was inactivated the next day or after 48 hours if 24 hours was not adequate for protein digestion.

To increase the aqueous layer 300 µl of high Tris EDTA (TE) buffer was added and followed by spinning the samples with an equal volume (600 µl total) of phenol-chloroform at 13,000rpm for 5 minutes. Then the aqueous layer was transferred to a fresh tube, and the phenol-chloroform step was repeated for 2 more times except in the last time chloroform only was added to the sample.

For DNA precipitation, 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol were added. After thorough mixing, the tube was left in a freezer at -20°C for 30 minutes. The tube was then spun for 10 minutes at 13,000 rpm. The supernatant was discarded, and the pellet was washed with 1 ml 70% alcohol and spun at 13,000 rpm for 10 minutes. The supernatant was discarded carefully, and the pellet dried. The pellet was then re-suspended in 25 µl low TE buffer and incubated at 54°C for 60 minutes to allow the DNA to solubilize.

2.3 PCR and DNA Sequencing

PCR was carried out in a final volume of 20 µl, containing 13.4 ul of dH2O, 2 µl of 10× buffer, 0.8 ul of 50 mM $MgCl^2$, 2 µl of 1.2 mM deoxynucleoside triphosphate, 0.4 µl of 10 mM of each primer, and 0.04 µl of 5 U/ µl Taq polymerase (Invitrogen) and 1 µl of 200 ng of DNA. The cycling conditions comprise a denaturation step at 95◦C for 2 min, followed by 40 amplification cycles at 95◦C for 30 s, 55◦C for 45 s and 72◦C for 90 s, and a final extension at 72◦C for 7 min.

Two types of PCR were carried out. First, an allele-specific reaction where the 4G/5G-promoter polymorphism was ascertained by the following primers: 1) insertion 5G allele: 5'-GAC ACG TGG GGG AGT CAG-3' and 2) deletion 4G allele: 5'-GGA CAC GTG GGG AGT CAG-3', each in combination with 3) a common downstream primer 5'- ACC TCC ATC AAA ACG TGG AA -3'. These primers are from the paper by Bedaiwy et al. [141]. The 4G and 5G forward primers both extend over the polymorphism, with the only difference being at the polymorphism site.

Electrophoresis of the PCR products was performed on agarose gel and visualized by staining with ethidium bromide, followed by ultraviolet transillumination. The genotype (4G/4G, 4G/5G, 5G/5G) was determined by whether a band is seen with the allele-specific forward primer (4G or 5G). For example, the 4G/4G genotype would be identified by only a band from the 4G specific primer; the 4G/5G genotype identified by a band from both the 4G specific primer and 5G specific primer; and the 5G/5G genotype identified by a band from only the 5G specific primer.

Second, PCR was performed for direct sequencing. This involved a positive control upstream primer 5'-CCAGACAAGGTTGTTGACACA-3' and the same common downstream primer. The 299 bp PCR product was then sent to DNA Sequencing Core Facility in CFRI for sequencing using ABI Prism 3130xl Genetic Analyzer.

For troubleshooting, I tried to design a second set of new primers for sequencing. 1) Primer pair 1: Forward primer: 5'-GACAAGGTTGTTGACACAAG-3', and Reverse primer: 5'-TCTTTCCCTCATCCCTGC-3'. 2) Primer pair 2: Forward primer: 5'-AGGTTGTTGACACAAGAGAG-3' and Reverse primer: 5'-TTTCCCTCATCCCTGCC-3'. These primers are closer to the 4G polymorphism with the hope that a shorter amplicon would provide more accurate sequencing findings.

I also explored DNA purification before sequencing. However, most of the DNA was lost when I tried the silica membrane method (QIAquick Kits) to purify the PCR product. Another method using a commercial kit (ExoSAP-IT PCR Cleanup, Biolynx, Brockville, ON) was utilized for

purifying PCR products trying to get a clean DNA sequence. This method utilizes two hydrolytic enzymes, Exonuclease I (Exo I) and Shrimp Alkaline Phosphatase (SAP). Exo I enzyme degrades residual single-stranded primers and any extraneous single-stranded DNA produced in the PCR. SAP enzymes dephosphorylates the remaining dNTPs, and, thus inactivates them in the PCR reaction.

2.4 Validation of PAI-1 Antibody

Different concentrations of PAI-1 monoclonal antibody (TJA6, Cat# sc-59636 Santa Cruz Biotechnology, Dallas, Texas U.S.A) was used in positive control cells (human cytotrophoblast). However, there was no observed staining. We therefore, chose another PAI-1 antibody that has been used in several published papers [148] [149] (dilution 1:25, PAI-1 Antibody (C-9), Cat#sc-5297; Santa Cruz Biotechnology, Dallas, Texas U.S.A). This second PAI-1 antibody was positive in human cytotrophoblast cells.

We then proceeded to formally validate this antibody. HepG2 cells are known to express PAI-1. We validated the antibody by knockdown of the PAI-1 gene in HepG2 cells through utilizing siRNA. After that, immunocytochemistry was employed using PAI-1 antibody to compare PAI-1 expression in knocked down HepG2 cells with control cells. The antibody was validated when PAI-1 expression was reduced in knocked down cells compared to the control cells.

2.4.1 Cell Culture Preparation for Immunocytochemistry

Human liver hepatocellular carcinoma cell line (HepG2 cells) was kindly provided by Dr. Geoffrey L. Hammond (Department of Cellular and Physiological Sciences, UBC) Fresh DMEM (Dulbecco's Modified Eagle Medium) (Sigma-Aldrich, Oakville, Ontario, Canada) media supplemented with 10% fetal bovine serum (FBS) (HyClone) were used for cell culture. Culture medium was discarded every 2-3 days. For subculturing, when cells reached 80% confluence, they were rinsed with 1x PBS twice and then incubated with a pre-warmed trypsin-EDTA solution for 5 minutes at 37°C to allow cells to be dispersed. After that trypsin was deactivated by adding a double volume of growth medium (including 10% FBS). Cultures were incubated at 37°C in humidified incubator with 5% CO2.

2.4.2 Small Interfering RNA (siRNA) Transfection

Human SERPINE1 siRNA (Qiagen Flextube hs siSERPINE1 (Cat no: GS5054) (5nM), Lipofectamine RNAiMAX Reagent (Invitrogen), and Opti-MEM I Reduced Serum Medium (Invitrogen) were used for siRNA transfection. Cells were maintained in 3.5 cm dish in DMEM containing 10% FBS with 50 % confluence. Transfections were performed with Opti-MEM I Reduced Serum Medium and Lipofectamine RNAiMAX Reagent according to the manufacturer's guidelines. AllStars Negative Control siRNA (Qiagen) was used for control. The cells were collected for immunocytochemistry and RNA extraction after 3 days of siRNA transfection. Knockdown efficiency was assessed by RT-qPCR.

2.4.3 Immunocytochemistry

The immunocytochemistry procedure for HepG2 cells was performed as mentioned below in the immunohistochemistry section (section 2.5) except for minor changes. PAI-1 knocked down and negative control siRNA HepG2 cells were grown on cover slips to reach approximately 70-80% confluence. The cover slips were transferred to a well plate then ice cold methanol was added for fixation. After that 0.1% Tergitol-type NP-40 diluted in PBS was added to each well to permeabilize the cells, prior to immunochemistry (section 2.5).

2.4.4 RNA Extraction

The TRIzol Reagent (Ambion, Cat#15596026) was used for RNA extraction from HepG2 cells. One ml of Trizol was added to the cell culture dish, then all dish content was transferred to the Eppendorf tubes and kept in -80 °C until RNA extraction process. The tubes were then vortexed and incubated at room temperature for 5 minutes. For separation, 200 µl of chloroform was added to each tube and the tubes were vortexed again followed by centrifugation at 13,000g for 15 minutes. The aqueous phase was transferred carefully into fresh tubes followed by mixing the samples with 0.5 ml of isopropyl alcohol for precipitation.

The samples were incubated at room temperature for 15 minutes and then centrifuged at 13,000g for 15 minutes at 4 °C. The pellet was clearly seen, and the supernatant was eliminated. At least one ml of 75% ethanol was added to each tube to wash the RNA. Then, samples were centrifuged at 7500g for 5 minutes at room temperature. Then, the rest of the ethanol was removed. Finally, RNA 25 µl buffer ATE elution solution (Qiagen, Germany) was added the tubes. A NanoDrop-

1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) was used to measure the amount of RNA isolated.

2.4.5 Reverse Transcription

The High Capacity cDNA RT kit (Applied Bio systems, Cat# 4368814, Luithaunia) was used to make complementary DNA (cDNA). 1.5 µg of total RNA was used to prepare 10 µL RNA solution per 20-µL reverse transcription reaction. The master mix was prepared as follows: 2 µL of 10× RT buffer, 2 µL of 10x random primer, 0.8 µL of 25× dNTP, 4.2 µL DNAse/RNAse free water and 1 µL of Reverse Transcriptase. After that, RNA solution was added to the master mix and then processed in Eppendorf - Mastercycler Gradient (Eppendorf, Hamburg, Germany) according to the following cycles: 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes, and 4 °C for 60 minutes. The mixture was then stored at -20 °C freezer until Reverse Transcription Quantitative Real Time Polymerase Chain Reaction (RTqPCR).

2.4.6 Reverse Transcription Quantitative Real Time Polymerase Chain Reaction (RTqPCR)

RT-qPCR was used to assess PAI-1 knockdown efficiency in HepG2 cells. Each 20 µl reaction contained 10µl of 2×Power SYBR Green PCR Master Mix (Applied Bio systems, Cat# 4367656, Luithaunia), 80 ng cDNA and 300 nM of each specific primer. The reaction was 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus

ΔCq has a slope < |0.1|. The comparative CT ($2^{-\Delta\Delta CT}$) method with GAPDH as the reference gene was used to calculate PAI-1 knockdown efficiency. Primers used for gene amplification were as follows:

PAI-1 Fwd primer: 5'-CCTCAGGAAGCCCCTAGA-3'

PAI-1 Rvs primer: 5'-TGGAGAGGCTCTTGGTCT-3'

GAPDH qRT Fwd primer: 5'-GAGTCAACGGATTTGGTCG-3'

GAPDH qRT Rvs primer: 5'-GACAAGCTTCCCGTTCTCAG-3'.

2.5 Immunohistochemistry

After the PAI-1 antibody was validated, immunohistochemistry was performed for FFPE blocks from the cohort of deep infiltrating endometriosis, ovarian endometrioma, superficial endometriosis, and uterine endometrium (Aim 2); and from the Study Group (tender endometriosis with deep dyspareunia) and the Control Group 1 (non-tender endometriosis without deep dyspareunia) (Aim 3).

Formalin Fixed Paraffin Embedded (FFPE) endometrial tissue was sectioned at 4 μm slices. Immunohistochemistry was carried out as follows. The endometrial sections were de-paraffinized three times in 100% xylene for 3 min each. The sections were rehydrated in gradient ethanol solutions (100%, 95%, 80% and 50%) for 3 min each followed by incubation in Dako preheated antigen retrieval reagent (pH=6.0) for 30 min. After incubation with Dako Dual Endogenous Enzyme Block for 10 min, slides were incubated with anti-PAI-1 antibody at 4°C overnight. After

incubation with secondary antibody (Dako EnVision + Dual Link System-HRP) at room temperature for 30 min, the sections were developed with diaminobenzidine solution (Liquid DAB Chromogen System (Dako, Cat# K3468) and counterstained with Harris hematoxylin for 2 min (Sigma). Then, slides were dipped briefly in 4% acetic acid solution and then in bluing reagent (1% lithium carbonate) before being mounted.

2.6 Histoscore (H-SCORE)

The results of immunohistochemical staining for PAI-1 in endometriosis glands and stroma were semiquantitatively evaluated using the Histoscore method (H-score). Two observers who were blinded to the case scored the slides on a multi-headed microscope (U-MDO10B3, Olympus BX51, Olympus America, Melville, NY, USA).

The area of endometriosis epithelium and stroma was scanned in each slide at low power first (x10) and then analyzed with a magnification of x40 (HPF) to evaluate the staining intensity and estimate the proportion of positive cells in that fixed area. PAI-1 H-score was independently determined by each reviewer using the staining intensity scored on a four-tiered scale (negative = 0, low intensity positive staining = 1, moderate intensity positive staining = 2, and strong intensity positive staining = 3). The percentage of positive cells at each staining intensity level were calculated in each fixed field, and each stained section was given a histochemical score calculated by the formula: [1 × (% positive cells) + 2 ×(% positive cells) + 3 ×(% positive cells)] [148]. The mean score from the two observes was calculated and entered in the final analysis.

2.7 Statistical Analysis

2.7.1 Histoscore Analysis

For the cohort of deep infiltrating endometriosis, ovarian endometrioma, superficial endometriosis, and uterine endometrium (Aim 2), we used the Kruskal-Wallis test followed by the Mann-Whitney test for pairwise comparison. For the Study Group and Control Group 1 (Aim 3), we used the Mann-Whitney test. In both analyses, we compared the PAI-1 H-score in the endometriosis glandular epithelium and the stroma, separately. Also, using linear regression, we investigated the correlation between PAI-1 H-score and deep dyspareunia. Statistical inference was guided by p-values < 0.05 and 95% confidence intervals. All statistics was performed using SPSS 22.0TM.

Chapter 3: PAI-1 (SERPINE1) Genotyping (Aim 1)

3.1 Rationale

Plasminogen activator inhibitor-1 (PAI-1), or *SERPINE1*, is a serine protease inhibitor (serpin) encoded by the human *SERPINE1* gene. *SERPINE1* is a major inhibitor of fibrinolysis, a process that prevents blood clots from growing and becoming problematic. The *SERPINE1* gene has a 4G allele, which involves an insertion-deletion polymorphism in the promoter 675bp upstream from the start codon (rs1799768) [140]. This polymorphism results in an allele with four sequential guanosines (4G), rather than five sequential guanosines (5G), and the 4G allele is associated with increased expression of *SERPINE1*[140]. It was previously reported that the *SERPINE1* 4G allele was associated with endometriosis pain in a sub-analysis [73]. Furthermore, increased *SERPINE1* expression from the 4G allele and associated hypofibrinolysis would allow persistence of the local fibrin matrix that could act as a scaffold for nerve fiber formation [128]. Thus, we propose that the *SERPINE1* 4G allele leads to increased *SERPINE1* expression, which in turn leads to local nerve formation in cul-de-sac (Zone II of the pelvis) endometriosis, resulting in deep dyspareunia (Figure 3.1).

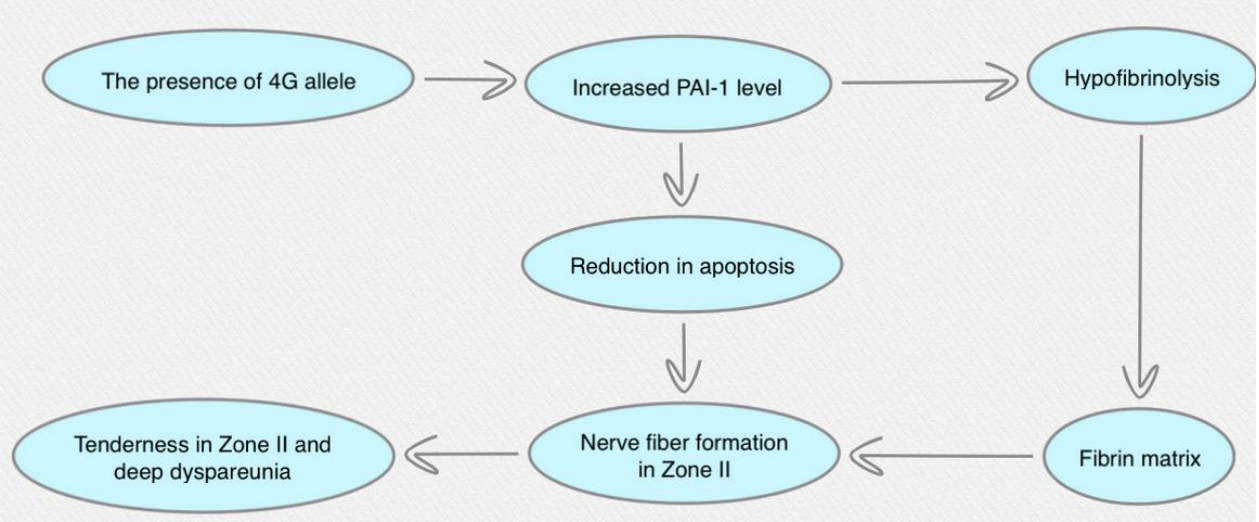

Figure 3.1: The proposed mechanism of PAI-1 (SERPINE1) role in nerve fiber formation in endometriosis.

3.2 Sample Characteristics

For Aim 1, laparoscopic surgeries during the period of 2011 to 2013 were reviewed, as we have previously described [73]. Endometriosis tissue was excised from the posterior compartment (cul-de-sac) and histologically confirmed on pathology. Included cases were reviewed for these variables: patient-reported deep dyspareunia (0-10), posterior compartment tenderness on endovaginal ultrasound assisted pelvic exam, and other clinical variables (age, hormonal suppression, the amount of histological endometriosis, deep vs. superficial endometriosis, and chronic pelvic pain severity).

The cul-de-sac corresponds to Zone II of the pelvis as published by Bedaiwy et al. [149] (Figure 3.2), which was adopted by the World Endometriosis Research Foundation for the Endometriosis Phenome and Biobanking Harmonisation Project [150]. Anatomically, Zone II is the midline posterior zone of the pelvic cavity, between the uterus and rectum (the cul-de-sac peritoneal fold, which is limited by the uterosacral ligaments bilaterally) (Table 3.1).

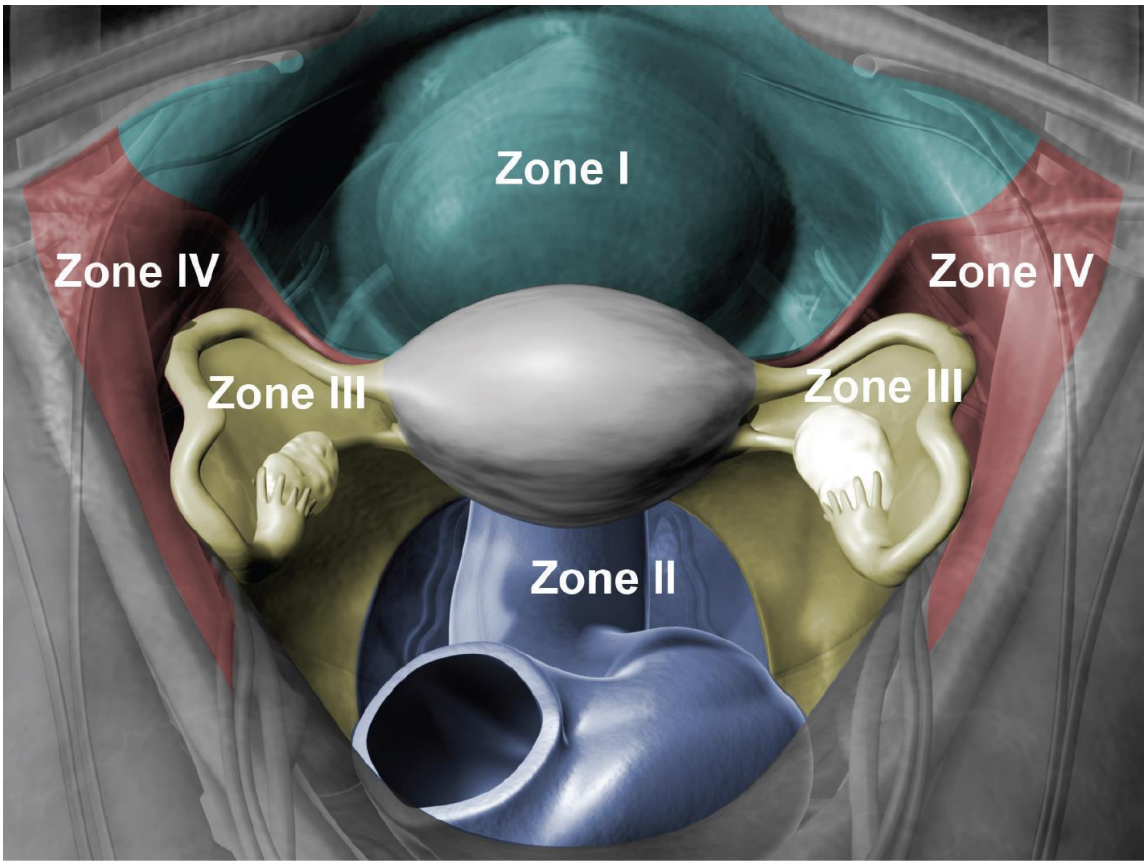

Figure 3.2: An illustration showing Zone II and its location in the pelvic cavity (from Bedaiwy et al. [149]).

Zone	Boundaries	Contents
Zone I	Midline anterior abdominal cavity limited by the round ligaments bilaterally	(1) Uterine dome and anterior surface (2) Anterior surface of the broad ligaments (3) Bladder dome (4) Internal ring and inferior epigastric vessels
Zone II	Midline posterior zone of the abdominal cavity limited by the uterosacral ligaments bilaterally	(1) Uterine dome and posterior surface (2) Pouch of Douglas (3) Rectovaginal septum (4) Sigmoid colon (5) Presacral peritoneum
Zone III	Lateral pelvic sidewalls limited by the uterosacral ligament and the adnexae and infundibulopelvic ligaments	(1) Fallopian tube and ovary (2) Posterior surface of the broad ligament (3) Ovarian fossa (4) Vessels and ureter
Zone IV	Pelvic sidewall limited by the round ligament, adnexae and infundibular ligament, and external iliac vessels	(1) External iliac vessels (2) Ilioinguinal nerve

Table 3.1: Summary of anatomical boundaries and the content of each pelvic zone (from Bedaiwy et al. [149]).

For Aim 1, we used two groups of patients from our previously published paper [73]. The Study Group (n = 21) (tender endometriosis) consisted of women meeting the following criteria: a) presence of deep dyspareunia; b) tenderness of the right, central, or left Zone II on endovaginal ultrasound assisted pelvic exam; c) histologically confirmed endometriosis of the corresponding right/central/left Zone II at laparoscopy; d) which was associated with a higher density of nerve bundles (Figure 3.3). For example, a patient in the Study Group may have deep dyspareunia, a tender left uterosacral, and histologically confirmed left uterosacral endometriosis.

Control Group (1) (n = 16) (non-tender endometriosis) consisted of women meeting the following criteria: a) absence of deep dyspareunia (patients were referred for infertility alone); b) absence of Zone II tenderness on endovaginal ultrasound assisted pelvic exam; c) histologically confirmed endometriosis of Zone II; and d) which was associated with a lower density of nerve bundles. For example, a patient in Control Group may have no deep dyspareunia, and no tenderness, but histologically confirmed right uterosacral endometriosis (Figure 3.3). The purpose of Control Group was to identify Zone II endometriosis that was non-tender on exam, and that did not manifest as deep dyspareunia, in order to determine whether PAI-1 4G allele and PAI-1 expression level were specific to tenderness/deep dyspareunia and higher density of nerve bundles.

The mean (±SD) age of the Study Group (n=21) was 29.6 (± 5.92) years compared with 34.06 (± 3.34) years in the controls (n=16) (p = 0.023).

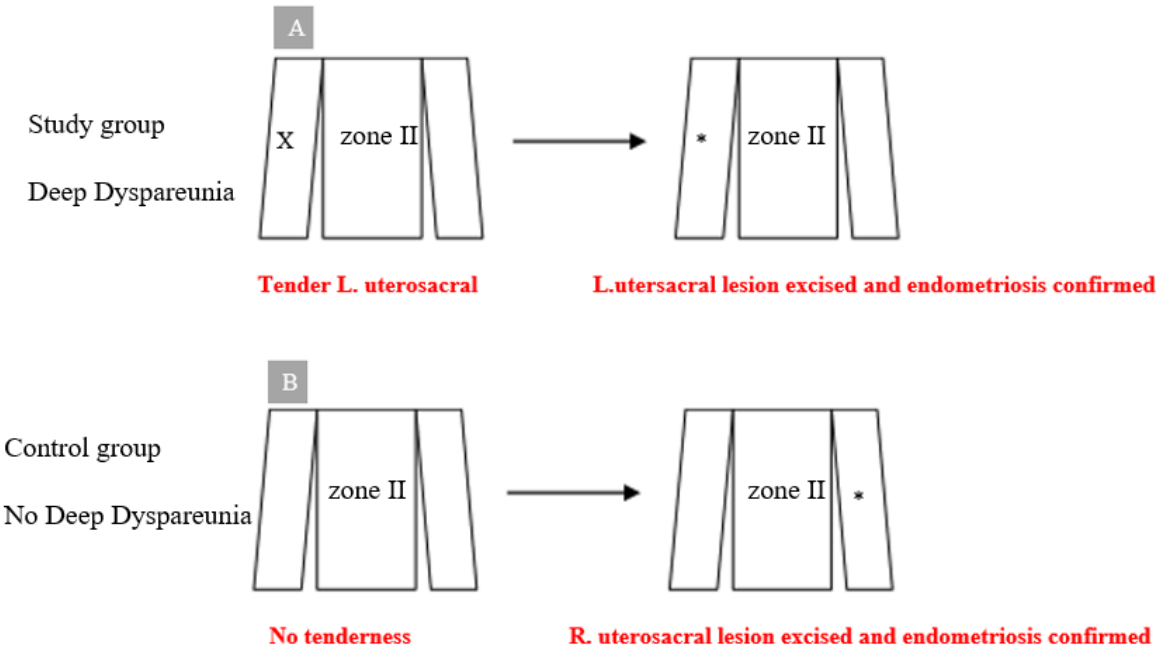

Figure 3.3: An illustration of cases criteria.

A. Study Group (tender endometriosis): Deep dyspareunia. B. Control Group (non-tender endometriosis): No deep dyspareunia (infertility).

For the SERPINE1 4G allele, routinely formalin-fixed paraffin-embedded (FFPE) endometrial tissue specimens were retrieved from the archives. Sectioning of paraffin blocks at 20 μ scroll thickness was performed in the pathology (BC Women Hospital). Genomic DNA was prepared from these FFPE blocks of the endometriosis patients using the Phenol-chloroform (PC) extraction method. As mentioned in chapter 2, two types of PCR were carried out. First, an allele-specific reaction, with primers from the paper by Bedaiwy et al. [141] (Figure 3.4). The 4G and 5G forward primers both extend over the polymorphism, with the only difference being at the polymorphism site. Second, PCR for sequencing, which involved a positive control upstream primer and the same common downstream primer (Figure 3.4). The 299 bp PCR product was then sent to DNA Sequencing Core Facility in CFRI for sequencing DNA.

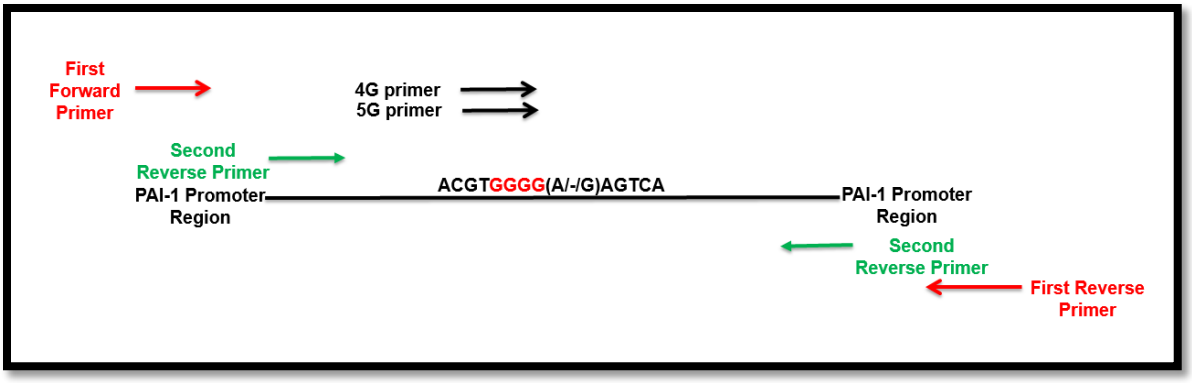

Figure 3.4: A diagram showing different primers have been utilized for amplifying rs1799768.

The allele-specific primers are denoted by 4G or 5G, with a common downstream primer (first reverse primer). For sequencing, I used a positive control upstream primer (first forward primer) and the common downstream primer (first reverse primer). The second forward and reverse primers are discussed later.

3.3 Results and Troubleshooting

For SERPINE1 genotyping, genomic DNA was successfully extracted from FFPE blocks among 32 cases (Figure 3.5). PCR was performed for 17 cases (7 Study Group samples and 5 Control Group samples, as well as 5 test samples), in addition to 2 DNA control samples (DNA extracted from blood) from Robinson lab. For the allele-specific PCR, all samples appeared to be heterozygous 4G/5G on the agarose gel (Figure 3.6). However, this was discordant with the sequencing results (see below). We suspect that, under the current conditions, the 4G and 5G allele-specific primers were binding regardless of the allele present.

For PCR for sequencing, we sent 14 PCR products (7 PCR products from Study Group, 5 PCR products from Control Group, and 2 DNA control samples from the Robinson lab) to the DNA Sequencing Core Facility in CFRI for DNA sequencing using ABI Prism 3130xl Genetic Analyzer. Based on these results, 6 cases in the Study Group were classified as 4G/4G genotype (Figure 3.7). However, closer review showed possible mis-sequencing and jumbled peaks, rounded and not too well resolved peaks in some cases (Figure 3.8). Other cases showed multiple mis-sequencing that we could not delineate the polymorphism site (Figure 3.9). It is clear that with this FFPE extracted DNA, the conditions of sequencing still needed to be optimized.

For troubleshooting, we tried to design a second set of primers for sequencing. 1) Primer pair 1: Forward primer: 5'-GACAAGGTTGTTGACACAAG-3', and Reverse primer: 5'-TCTTTCCCTCATCCCTGC-3'. 2) Primer pair 2: Forward primer: 5'-AGGTTGTTGACACAAGAGAG-3' and Reverse primer: 5'-TTTCCCTCATCCCTGCC-3'.

These primers are closer to the 4G polymorphism (Figure 3.4), with the hope that a shorter amplicon would provide more accurate sequencing findings. However, after sequencing 3 cases using the new primers, we could not delineate the polymorphism site.

We also explored DNA purification before sequencing. However, most of the DNA was lost when we tried the silica membrane method (QIAquick Kits) to purify the PCR product. Another method using a commercial kit (ExoSAP-IT PCR Cleanup, Biolynx, Brockville, ON) was utilized for purifying PCR products trying to get a clean DNA sequence. This method utilizes two hydrolytic enzymes, Exonuclease I (Exo I) and Shrimp Alkaline Phosphatase (SAP). Exo I enzyme degrades residual single-stranded primers and any extraneous single-stranded DNA produced in the PCR. SAP enzyme dephosphorylates the remaining dNTPs, and thus inactivates them in the PCR reaction. Using this method, sequencing results were somehow improved. Peaks seemed to be well defined and sharp, with nice spacing between them. There was little background interference present at the peak baselines (Figure 3.10). However, there were sequence artifacts from the same samples. That is, the same sample was found to yield two different readings when sequenced twice (Figure 3.11). These sequence alterations were not seen when we used the control DNA sample extracted from blood (Figure 3.11).

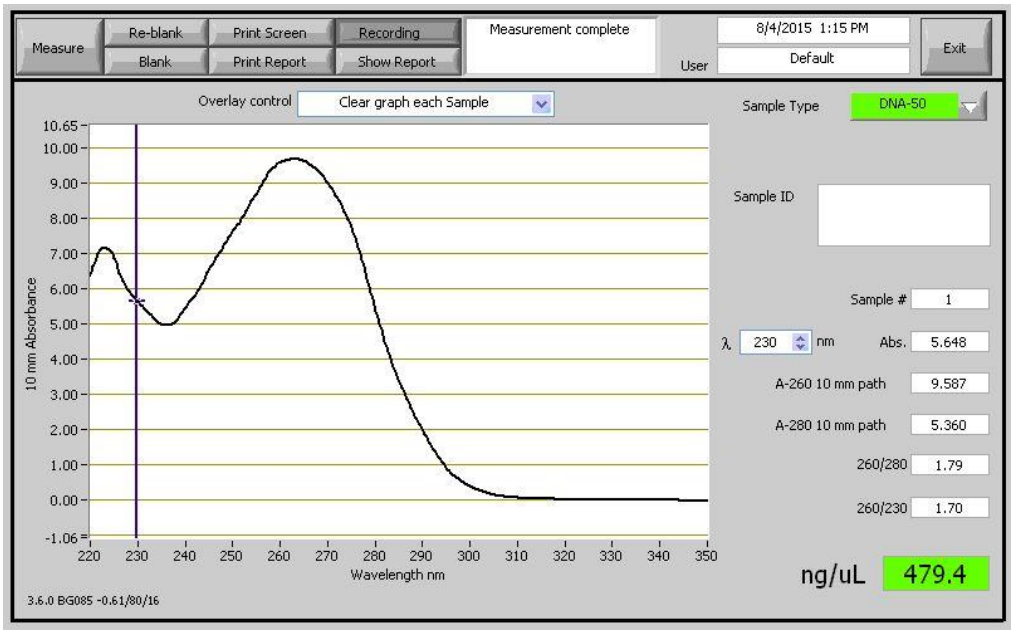

Figure 3. 5: Concentration of genomic DNA using PC extraction method from FFPE tissue.

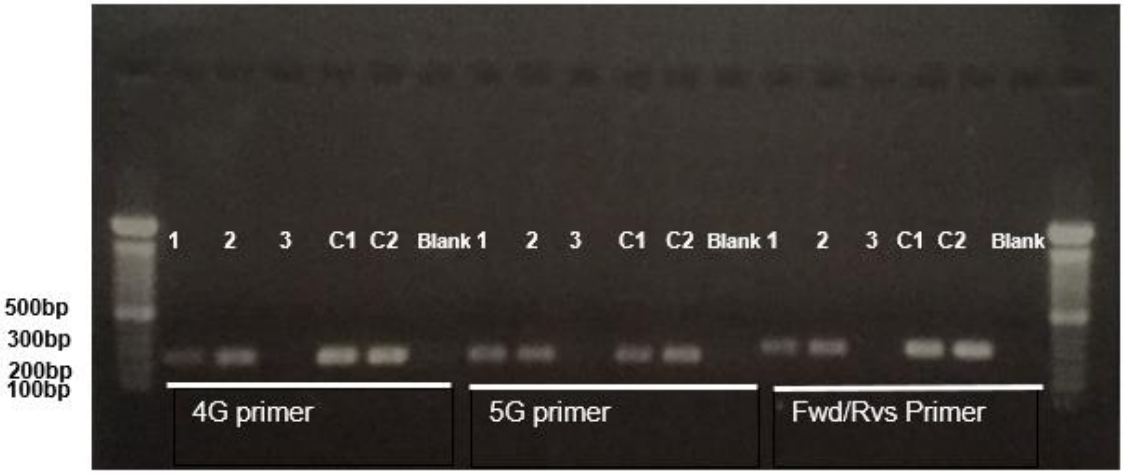

Figure 3.6: The 248 and 299 bp DNA fragments after PCR reaction.

Samples 1, 2 & 3 from study group while C1 and C2 are DNA controls from Robinson lab.

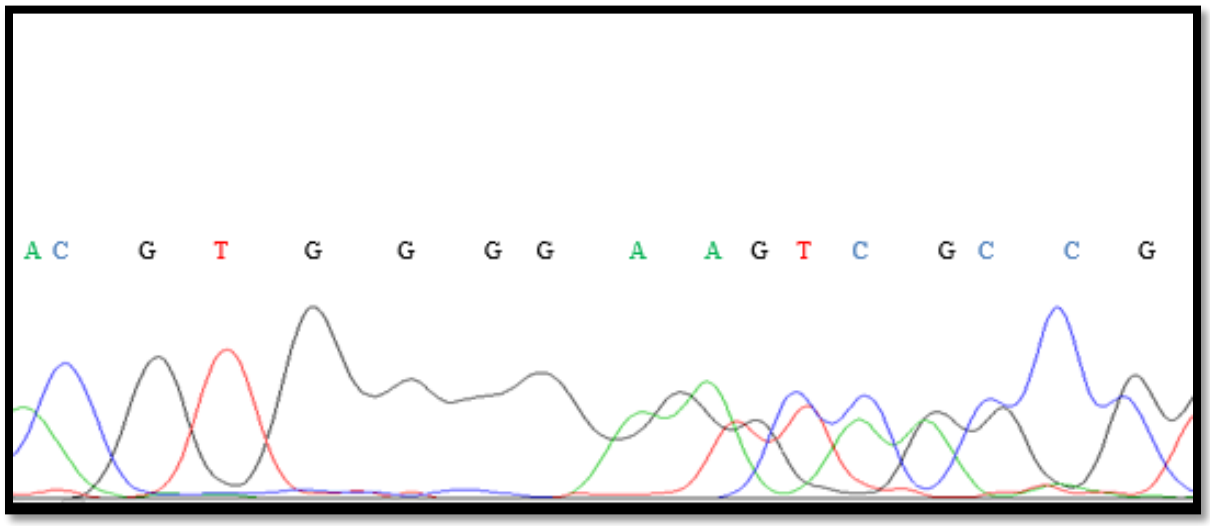

Figure 3.7: Results of sequencing rs1799768, seemed to be homozygous allele (4G/4G).

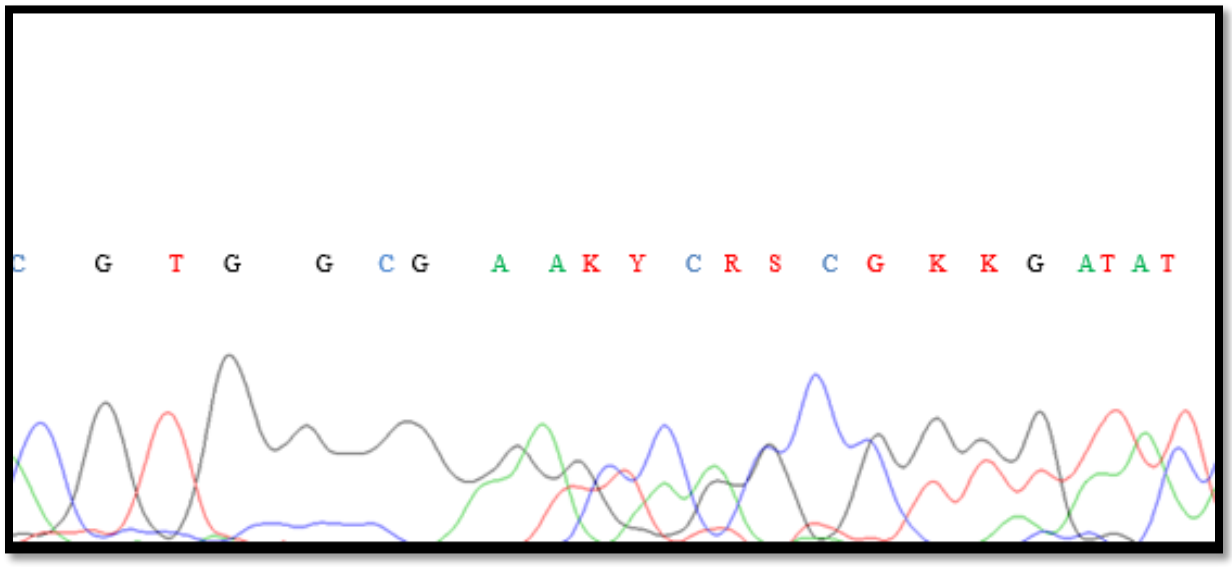

Figure 3.8: An example of sequencing rs1799768.

Sequencing results in which a 4G/4G genotype is inferred, but there is poor quality sequence with jumbled, rounded and poorly resolved peaks.

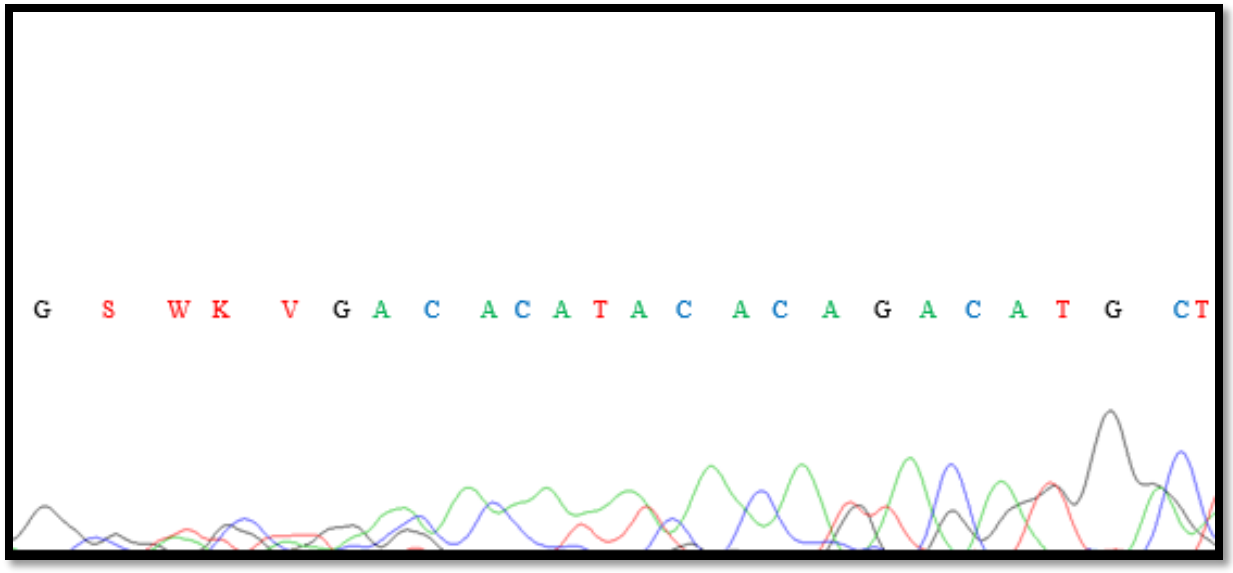

Figure 3.9: An example of poor quality sequence result in which the polymorphism site could not be delineated.

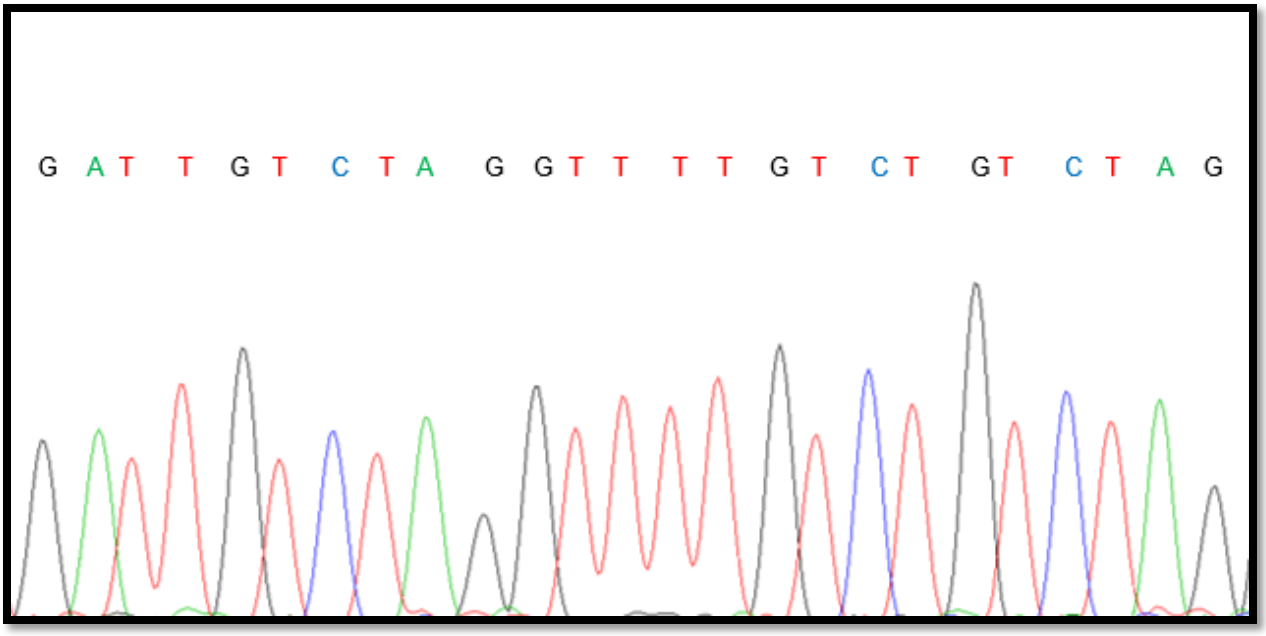

Figure 3.10: Results of sequencing rs1799768, reveals sequencing results were somehow improved with purification.

Peaks seemed to be well defined and sharp, with nice spacing between them. There was little background interference present at the peak baselines. However, we were not able to delineate the polymorphism site. The same sample seemed to be homozygous allele (4G/4G) in the previous sequencing (Figure 3.8).

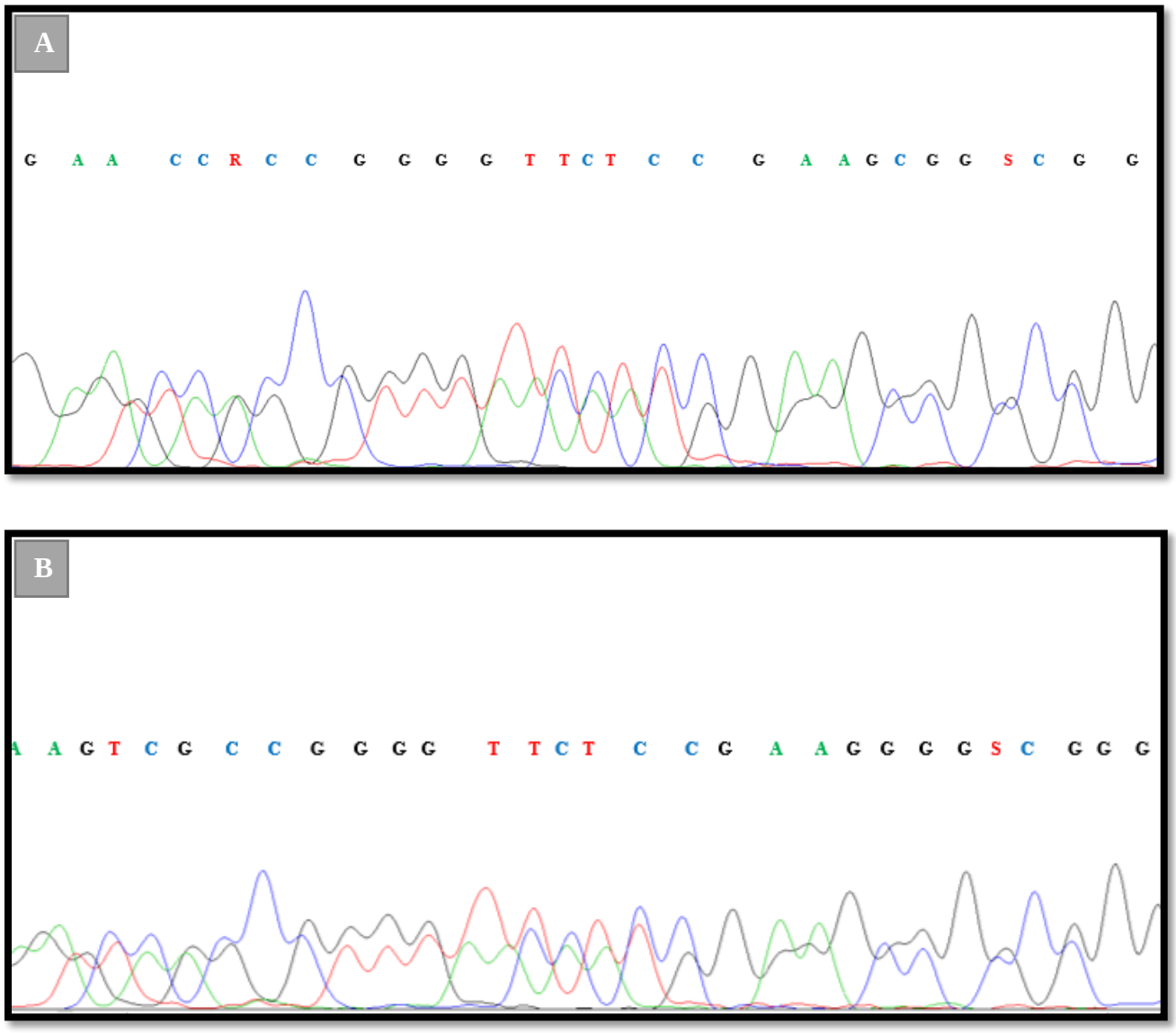

Figure 3.11: Minimal sequence alterations were seen when we used the control blood DNA sample.

The same sample was sequenced 2 times as control along with study group samples.

3.4 Discussion

DNA is susceptible to degradation when in formalin-fixed paraffin-embedded tissues. Another major problem related to FFPE DNA is the presence of sequence artifacts, i.e., obvious sequence changes that are not found in the original sequence. Several studies have reported that the number of sequence alterations seen in FFPE tissues is greater than that in matched frozen tissues [154] [155].

It is indeed challenging to distinguish true sequence changes from artefactual sequence changes. These limitations would cause increased risk of false-positive mutation calls [153]. For example, multiple novel mutations in epidermal growth factor receptor (EGFR) in formalin-fixed tissues DNA have been reported [154] that never been present in over 2000 fresh-frozen tissues, of non–small cell lung cancer [155]. Also, it has been reported that multiple sequence artifacts in the EGFR gene can present in the formalin-fixed lung tissues [159] [160].

Fixed human tissues could be difficult to analyze if formalin damages DNA. Formaldehyde, the major component of formalin, is a chemical compound that can induce many crosslinks between intracellular macromolecules such as protein and DNA [158]. The formaldehyde-induced crosslinks include protein–protein,protein–DNA,and DNA–formaldehyde adducts and interstrand DNA crosslinks. These crosslinks of DNA can affect the stability of DNA. Also, it has been reported that DNA extracted from formalin fixed, paraffin embedded tissues may be damaged but still readable [152], which might be the case in PAI-1 genotyping as some cases were readable (Figure 7 and 8). In particular, sequence artifacts can originate from damaged templates presenting

in FFPE DNA, physical abnormalities in the DNA, such as single- and double-strand breaks [151], or oxidative DNA damage during sample preparation [159]. Future troubleshooting will include optimizing $MgCl_2$ concentration, as it an important factor for taq polymerase activity, and increasing control samples (known sequence) to demonstrate PCR specificity. Also, optimizing annealing temperature could prevent non-specific binding for PCR.

The above challenges were magnified by our attempt to sequence a single-base insertion-deletion polymorphism (4G/5G). Due to these limitations, I proceeded to validate an antibody for PAI-1 (SERPINE1) and performed immunohistochemistry for PAI-1 (SERPINE1) expression in deep infiltrating endometriosis (Aim 2) and in endometriosis deep dyspareunia (Aim 3).

Chapter 4: PAI-1 and Deep Infiltrating Endometriosis (Aim 2)

4.1 Rationale

The plasminogen activator (PA) system has been implicated in tumor invasion, breaking down of anatomical barriers and the migration of tumor cells into normal adjacent host tissues. In addition to PAI-1 being associated with thrombosis/fibrosis (by inhibiting fibrinolysis), the idea that PAI-1 involved in cell invasion and migration was first suggested by Bajou et al. [114]. It was found that deficient PAI-1 expression in host mice prevented local invasion and tumor vascularization of transplanted malignant keratinocytes. When PAI-1 expression was obtained by intravenous injection of a replication-defective adenoviral vector expressing human PAI-1, invasion and associated angiogenesis were restored.

Moreover, Liu et al. investigated the importance of the urokinase (uPA)-plasmin system and PAI-1 in human lung cancer cell invasion. Polyclonal antibodies, which inhibit uPA and PAI-1 activities, were utilized for the highly invasive H292 cell line. It was found that invasive capacity was inhibited by antibodies to both uPA and PAI-1 in a dose-dependent manner. These data demonstrated that the uPA-plasmin system is important in promoting invasion into basement membranes. It seems that there should be a critical balance between uPA and PAI-1 for optimal invasiveness [115].

Using immunohistochemical staining, expression of PAI-1 was significantly higher in women with endometrial cancer stage III compared to stage I and II [116]. PAI-1 levels are elevated in many cancers including breast cancer [118], endometrial cancer [119] and lung cancer [120].

In summary, increased PAI-1 expression may be associated with thrombosis/fibrosis and tumor invasion. Therefore, I propose that PAI-1 expression will be increased in deep infiltrating endometriosis, which exhibits these qualities. Before assessing PAI-1 protein expression, we validated an antibody for PAI-1.

4.2 Sample Characteristics

Antibody validation was performed using HepG2 cells (hepatocellular carcinoma). These cells are known to express PAI-1. We validated the antibody by knockdown of the PAI-1 gene in HepG2 cells through utilizing siRNA. After that, immunocytochemistry was performed using PAI-1 antibody to compare PAI-1 expression in knocked down HepG2 cells with control cells. The antibody was validated when PAI-1 expression was reduced in knocked down cells compared to the control one.

After validation, immunohistochemical analysis using the validated monoclonal mouse PAI-1 antibody was performed to examine the expression of PAI-1 in formalin fixed paraffin embedded (FFPE) endometrial tissues from 30 women with different types of endometriosis: 10 cases with deep infiltrating endometriosis (DIE) (Group 1), 10 cases with endometrioma (ovarian endometriosis) (Group 2), and 10 cases with superficial endometriosis (Group 3). In addition, we examined 10 cases of uterine (eutopic) endometrium (from women with endometriosis) as a control (4 proliferative phase, 2 secretory phase, 4 unknown). One slide was excluded from the final analysis due to the absence of endometrial glands.

4.3 Results

4.3.1 PAI-1 Antibody Validation

To validate the mouse monoclonal antibody against PAI-1 for immunohistochemistry (IHC), human SERPIN1 siRNA was used for siRNA transfection to knockdown the PAI-1 gene in HepG2 cells. Also, another passage of HepG2 cells was treated with AllStars Negative Control siRNA. Knockdown efficiency was assessed by RT-qPCR (Figure 4.1). After that, we performed immunocytochemistry (ICC) of the PAI-1 antibody in both PAI-1 knocked down HepG2 cells and negative control siRNA HepG2 cells.

HepG2 cells had cytoplasmic immunostaining for PAI-1 (Figure 4.2). This cytoplasmic staining pattern was to be expected as it similar to another study [160]. Immunostaining was high in negative control siRNA HepG2 cells and low in PAI-1 knocked down HepG2 cells (Figure 4.2). This validated the PAI-1 antibody.

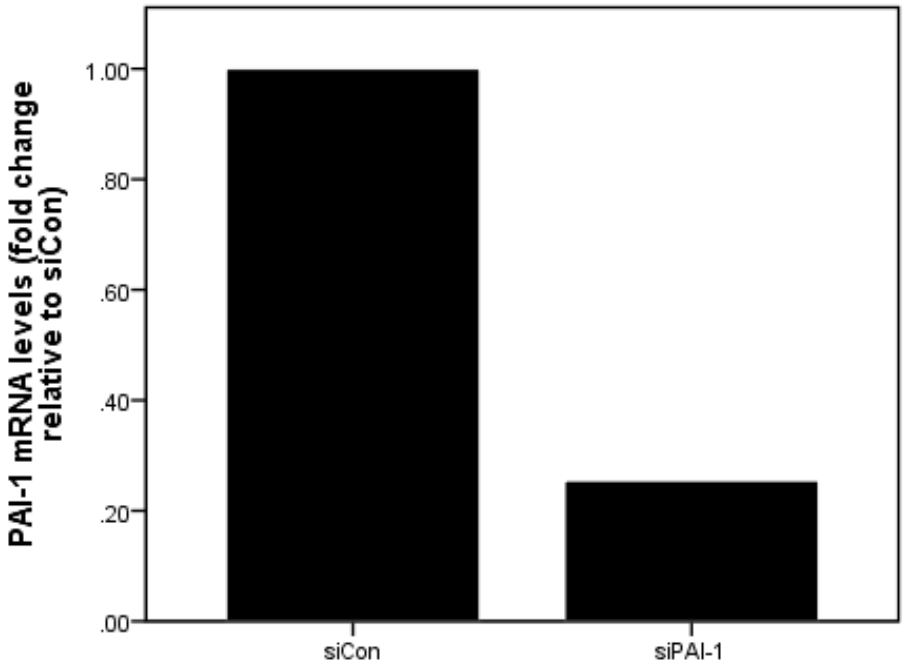

Figure 4.1: Knockdown of PAI-1 gene in HepG2 cells.

To validate the mouse monoclonal antibody against PAI-1 for immunohistochemistry (IHC), human SERPINE1 siRNA (siPAI-1) was used for siRNA transfection to knockdown PAI-1 gene in HepG2 cells. Also, another dish of HepG2 cells was treated with Control siRNA (siCon). PAI-1 mRNA levels were analyzed by RT-qPCR. The comparative CT ($2^{-\Delta\Delta CT}$) method with GAPDH as the reference gene was used to calculate PAI-1 knockdown efficiency.

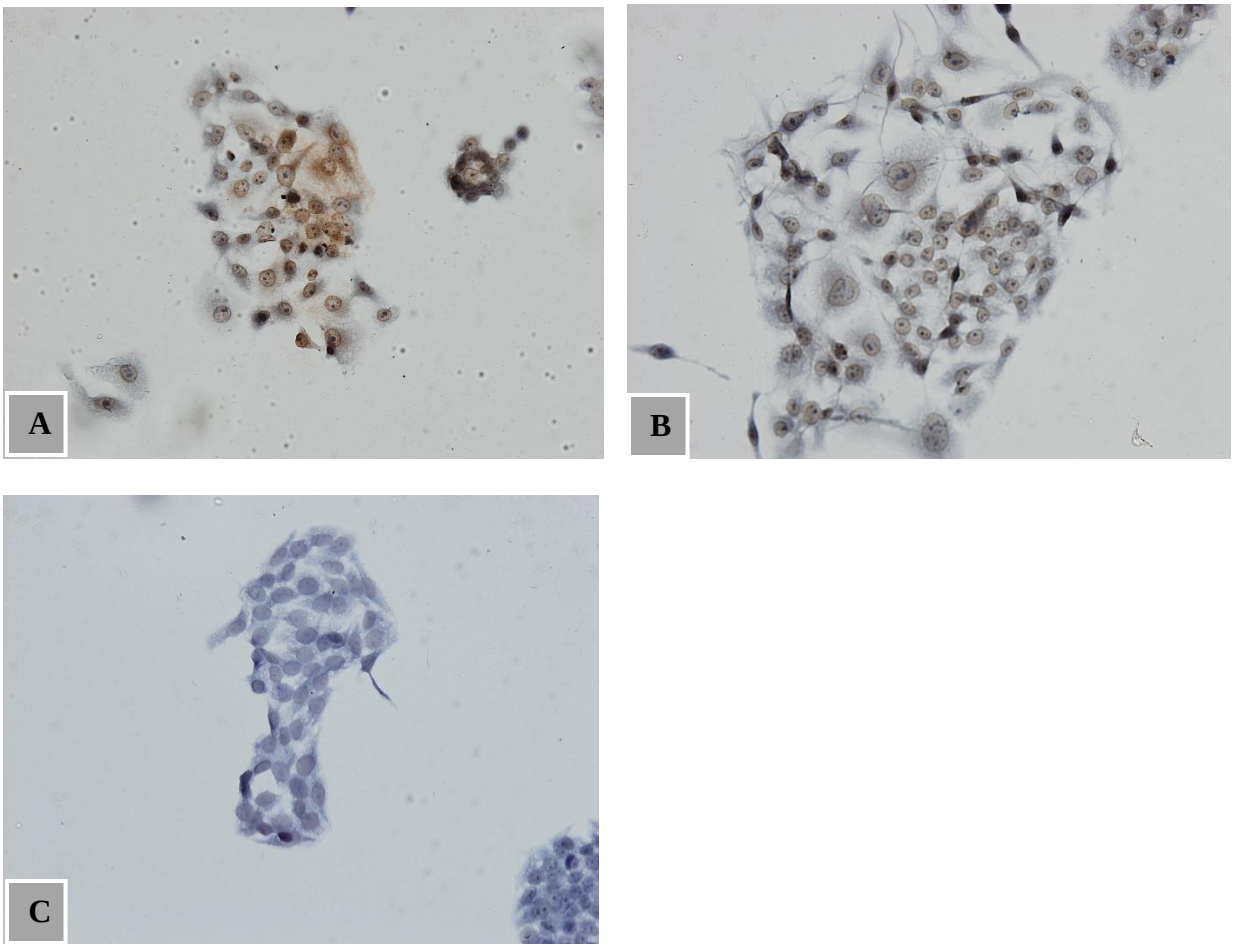

Figure 4.2: Immunostaining of PAI-1 in HepG2 cells.

Control siRNA HepG2 cells (A) and siPAI-1 HepG2 cells (B) were grown on cover slips to reach approximately 70-80% confluence. Cells were fixed and immunostained by the monoclonal PAI-1 antibody at dilutions 1:25, which showed staining in the control siRNA HepG2 cells but much less staining in the siPAI-1 knockdown HepG2 cells. The negative control (C) showed no primary antibody staining (20×).

4.3.2 PAI-1 Expression in Deep Infiltrating Endometriosis

Endometriotic tissue from the included women showed variable PAI-1 immunoreactivity. Estimating PAI-1 Histoscore (H-score) was performed for each slide as described before (Chapter 2). Staining intensity was determined as shown (Figure 4.3). We estimated PAI-1 immuno-intensity in endometriosis glandular epithelium and stroma.

Using the Histoscore, we found a difference between the groups in PAI-1 expression in glandular epithelium (Kruskal Wallis, $p = 0.005$) (Figure 4.4). On pairwise comparison, PAI-1 expression in glandular epithelium in the DIE group was significantly higher than in superficial endometriosis group ($p = 0.031$) and eutopic endometrium group ($p = 0.002$). However, this increased expression of PAI-1 in glandular epithelium in DIE was not significantly higher than in endometrioma ($p = 0.240$) (Figure 4.4 and 4.5). For endometrioma group, a significant difference was only seen in PAI-1 expression in GECs in endometrioma when compared to eutopic endometrium ($p=0.008$). In stroma cells, PAI-1 was higher in the DIE group compared to others (Kruskal Wallis, $p = 0.037$) (Figure 4.6). On pairwise comparison, the only statistically significant difference was seen in PAI-1 expression in the DIE group when compared to eutopic endometrium group ($p = 0.010$).

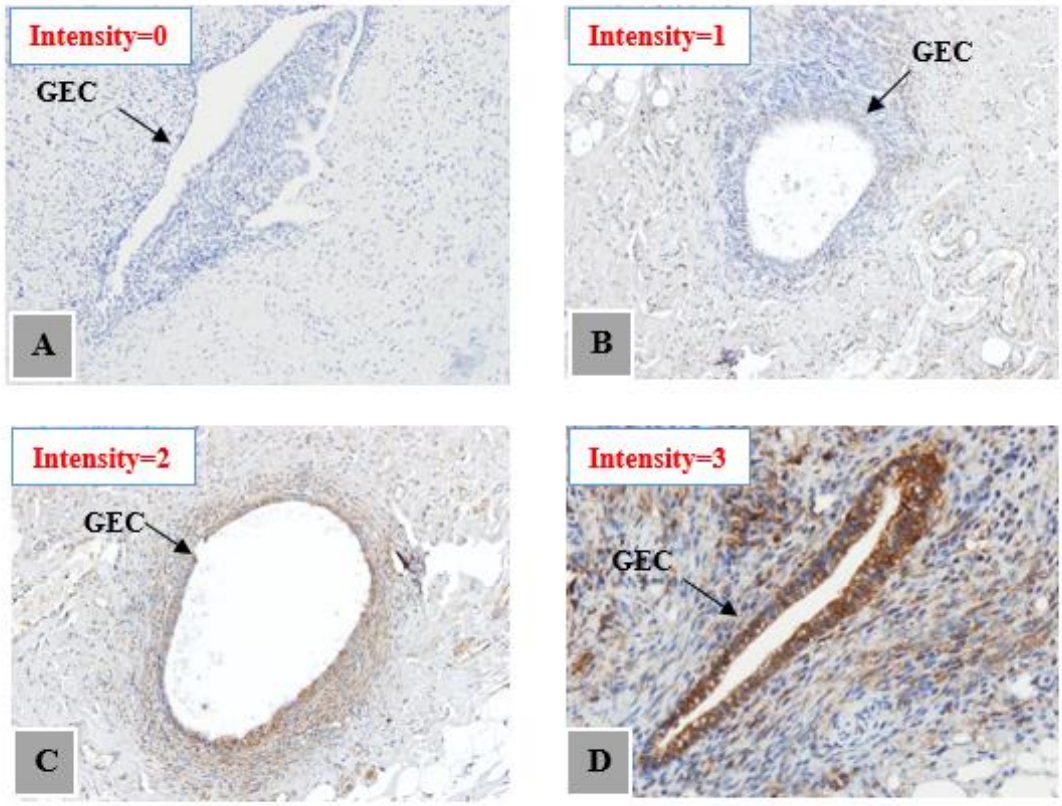

Figure 4.3: The PAI-1 immuno-intensity in women with superficial endometriosis.

PAI-1 H-score was independently determined by each reviewer using the staining intensity scored on a four-tiered scale (negative = 0) **(A)**, low intensity positive staining = 1 **(B)**, moderate intensity positive staining = 2 **(C)**, and strong intensity positive staining = 3 **(D)**). The percentage of positive cells at each staining intensity level was calculated in each fixed field, and each stained section was given a histochemical score calculated by the formula: [1 × (% positive cells) + 2 ×(% positive cells) + 3 × (% positive cells)] [148].

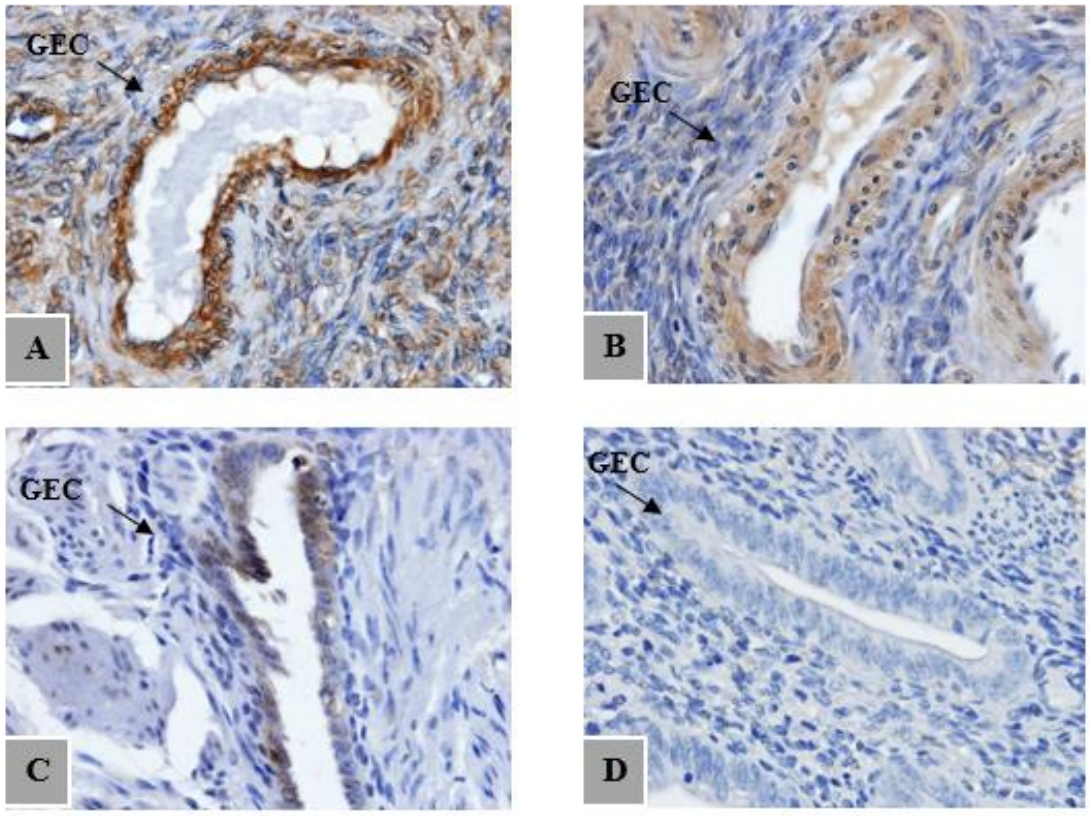

Figure 4.4: The difference in PAI-1 immuno-intensity in glandular epithelial cells (GECs) between the four groups : A) DIE. B) Endometrioma. C) Superficial endometriosis. D) Eutopic endometrium.

PAI-1 expression in glandular epithelium in the DIE group (A) was significantly higher than in superficial endometriosis group (C) ($p = 0.031$) and eutopic endometrium group (D) ($p = 0.002$). However, this increased expression of PAI-1 in glandular epithelium in DIE was not significantly higher than in endometrioma (B) ($p = 0.240$) (40×).

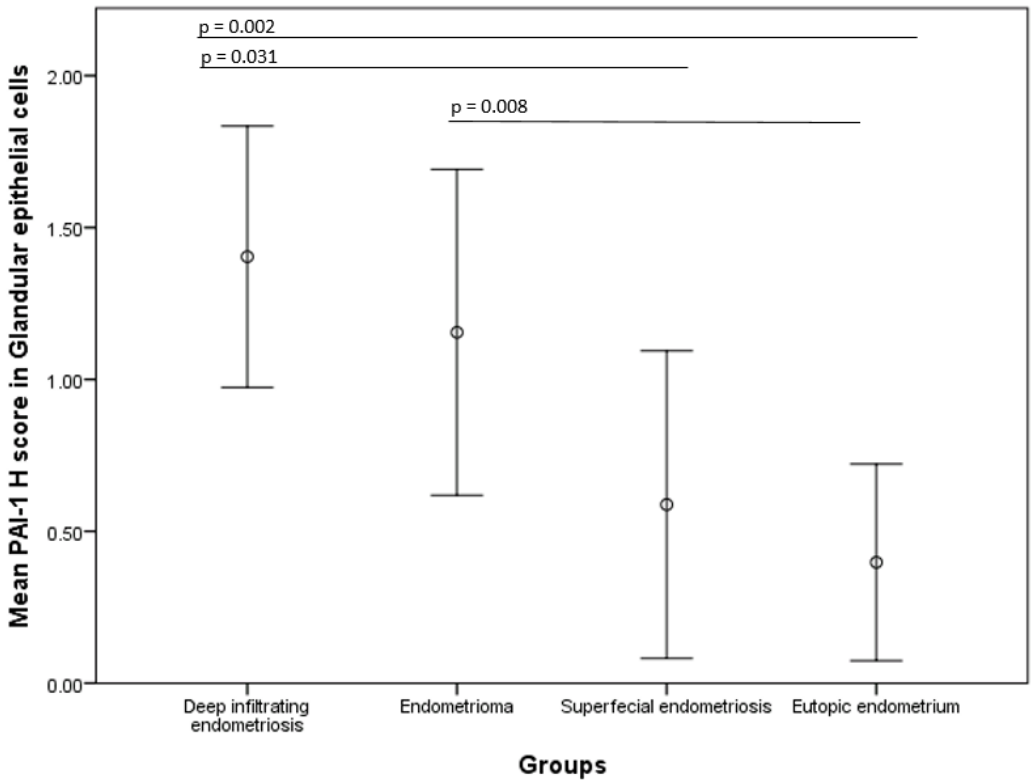

Figure 4.5: Analysis of PAI-1 immunoreactivity in the four groups: deep infiltrating endometriosis, endometrioma, superficial endometriosis, and eutopic endometrium using the Histoscore (H-score) in endometriosis glandular epithelial cells (GEC).

Mean H scores (± standard deviation [SD]) for the staining intensity of PAI-1 expression in the four groups. PAI-1 expression in GECs in the DIE group was significantly higher than in superficial endometriosis group ($p = 0.031$) and eutopic endometrium group ($p = 0.002$). However, this increased expression of PAI-1 in GECs in DIE was not significantly higher than in endometrioma ($p = 0.240$). For endometrioma group, a significant difference was only seen in PAI-1 expression in GECs in endometrioma when compared to eutopic endometrium ($p=0.008$).

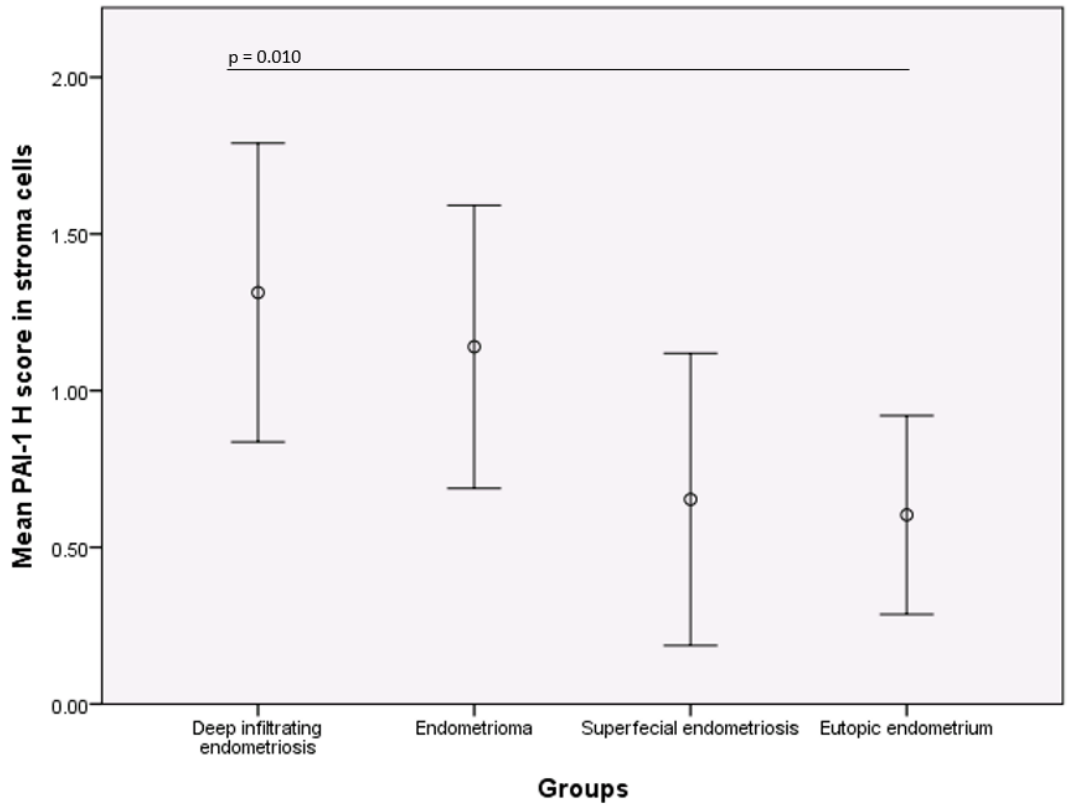

Figure 4.6: Analysis of PAI-1 immunoreactivity in the four groups: deep infiltrating endometriosis, endometrioma, superficial endometriosis, and eutopic endometrium using the Histoscore (H-score) in endometriosis stroma cells.

Mean H scores (± standard deviation [SD]) for the staining intensity of PAI-1 expression in the four groups. In stroma cells, PAI-1 was higher in the DIE group compared to others (Kruskal Wallis, p = 0.037). However, on pairwise comparison, the only statistically significant difference was seen in PAI-1 expression between the DIE group and eutopic endometrium group (p = 0.010).

4.4 Discussion

We found higher expression of PAI-1 in DIE group compared to other groups. In particular, PAI-1 was significantly higher in DIE group compared to superficial endometriosis group and uterine (eutopic) endometrium group where there is no invasion process. This observation makes sense, as invasion and fibrosis are characteristic of DIE, but not superficial endometriosis or uterine endometrium. There was no significant difference between DIE and ovarian endometrioma, possibly because invasion and fibrosis may also be features of endometriomas, which are cysts that invade the ovary and are associated with adhesions/fibrosis.

The concentration of u-PA and PAI-1 were higher in uterine endometrium from women with endometriosis than in uterine endometrium from controls. Also, both u-PA and PAI-1 were even higher in endometriotic tissue than in uterine endometrium. In this study, sample preparation included, endometriotic tissue samples, endometrial biopsy samples from healthy controls, and peritoneal fluid was obtained from women with endometriosis [106]. In a recent study, it was shown that endometriotic cells secreted more PAI-1 than uterine endometrial cells, and stromal cells (endometrial and endometriotic) secreted considerably more PAI-1 compared to epithelial cells [107].

To the best of our knowledge, the association between PAI-1 and deep infiltrating endometriosis has not been studied yet. However, our data is partially consistent with a published work where PAI-1 was significantly higher in women with endometrial cancer stage III compared to stage I and II [116]. PAI-1 levels are also elevated in many cancers including breast cancer [118],

endometrial cancer [119] and lung cancer [120]. This may indicate the involvement of PAI-1 in the invasive process in different diseases.

Strengths of our study are the use of the validated antibody. One limitation of the study is that it was conducted on retrospective cases. Also, only one representative slide per case was examined.

Having validated a PAI-1 antibody and shown increased PAI-1 expression in deep infiltrating endometriosis, we proceeded to assess PAI-1 expression and deep dyspareunia.

Chapter 5: PAI-1 and Deep Dyspareunia (Aim 3)

5.1 Rationale

The plasminogen activators (PA) have been reported to be involved in biological processes involving cell migration, tissue degradation, and remodelling. However, little is known about the expression and regulation of the PA system in the human endometrium in vivo. The PA system's involvement in controlling endometrial hemostasis and menstruation also is ambiguous.

Two specific inhibitors of PA system have been reported in humans, plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2). They are members of the serpin family and are expressed in the human endometrium [161] [105]. In particular, PAI-1 was detected in human and mouse uteri during implantation [162] [163]. It was also reported that t-PA activity and PAI-1 antigen were highly increased in the late secretory endometrium. However, PAI-1 activity could not be detected during menstruation [104]. In contrast, the concentration of PAI-1 was highly increased in endometriotic tissue compared to uterine endometrium [106].

In relation to nerve growth, increased PAI-1 expression and associated hypofibrinolysis could produce a local fibrin matrix that could act as a scaffold for nerve fiber formation [128]. In addition, there is some empirical evidence that increased PAI-1 expression helps to promote neuron survival, for example through a reduction in apoptosis [126]. It has been shown that astrocytes release PAI-1 to enhance neuron survival not only by activating the MAPK/ERK pathway but also by activating the c-Jun/ AP-1 pathway [126].

To the best of our knowledge, the association between PAI-1 and pain or neurogenesis in endometriosis has not been reported in the literature. Therefore, I hypothesized that the expression of PAI-1 would be higher in the Study Group (tender endometriosis with deep dyspareunia) compared to the Control Group (non-tender endometriosis without deep dyspareunia). I also hypothesized that PAI-1 expression would be correlated with nerve bundle density.

5.2 Sample Characteristics

Study Group and Control Group were described in Chapter 3 (n = 37). In summary, the Study Group includes women with cul-de-sac endometriosis, and deep dyspareunia and a tender cul-de-sac on examination (n = 21). The Control Group consists of women with cul-de-sac endometriosis, but without deep dyspareunia and without tenderness of the cul-de-sac on examination (n = 16). The sample sizes were smaller than in our original study [73], as we had to exclude some cases due to cauterized glands or absence of endometrial glands.

5.3 Results

Endometriotic tissue from the 37 women with endometriosis showed variable PAI-1 immunoreactivity. Estimating PAI-1 H-score was performed for each slide as described before (Chapter 2). Positive PAI-1 staining was seen in endometriotic glandular epithelial cells and stroma cells in both the Study Group and the Control Group.

There was no significant difference in PAI-1 immuno-intensity in glandular epithelium between the Study Group and the Control Group ($p = 0.068$) (Figure 5.1-5.2). In fact, the trend was towards

lower PAI immuno-intensity in the Study Group, in contrast to our hypothesis (Figure 5.2). Moreover, no statistically significant difference was seen in PAI-1 expression between the Study Group and Control Group for endometriosis stroma ($p = 0.25$). Correcting for deep vs. superficial endometriosis and hormone suppression vs. no hormone suppression did not change the results (data not shown).

Next, the Study Group and Control Group were combined, and we tested for a correlation between PAI-1 expression and severity of deep dyspareunia (rated 0-10 by the patients). Surprisingly, decreased PAI-1 expression in glandular epithelium was significantly correlated with increased deep dyspareunia ($r = - 0.352$, $p = 0.038$) (Figure 5.3), which again was in contrast to our hypothesis. However, there was no statistical correlation between PAI-1 expression and other parameters including nerve bundle density ($r = 0.069$, $p = 0.687$) (Figure 5.4), severity of chronic pelvic pain ($r = -0.230$, $p = 0.184$) (Figure 5.5), and age ($r = 0.164$, $p = 0.332$) (Data not shown). On regression analysis, (Log) PAI-1 expression significantly correlated with deep dyspareunia ($\beta = -0.378$, $p = 0.025$).

In addition to PAI-1 staining in endometriosis glands/epithelium, PAI-1 stained positive in the endothelium of blood vessels (BV), which strongly supports the sensitivity of the antibody, in serum, in nerve bundles (NB), in smooth muscle cells (SMCs), and also in macrophages (Figure 5.6). The expression of PAI-1 in nerves was confirmed using the pan-neuronal marker PGP9.5 slides, which were retrieved from the pathology archive (Figure 5.7).

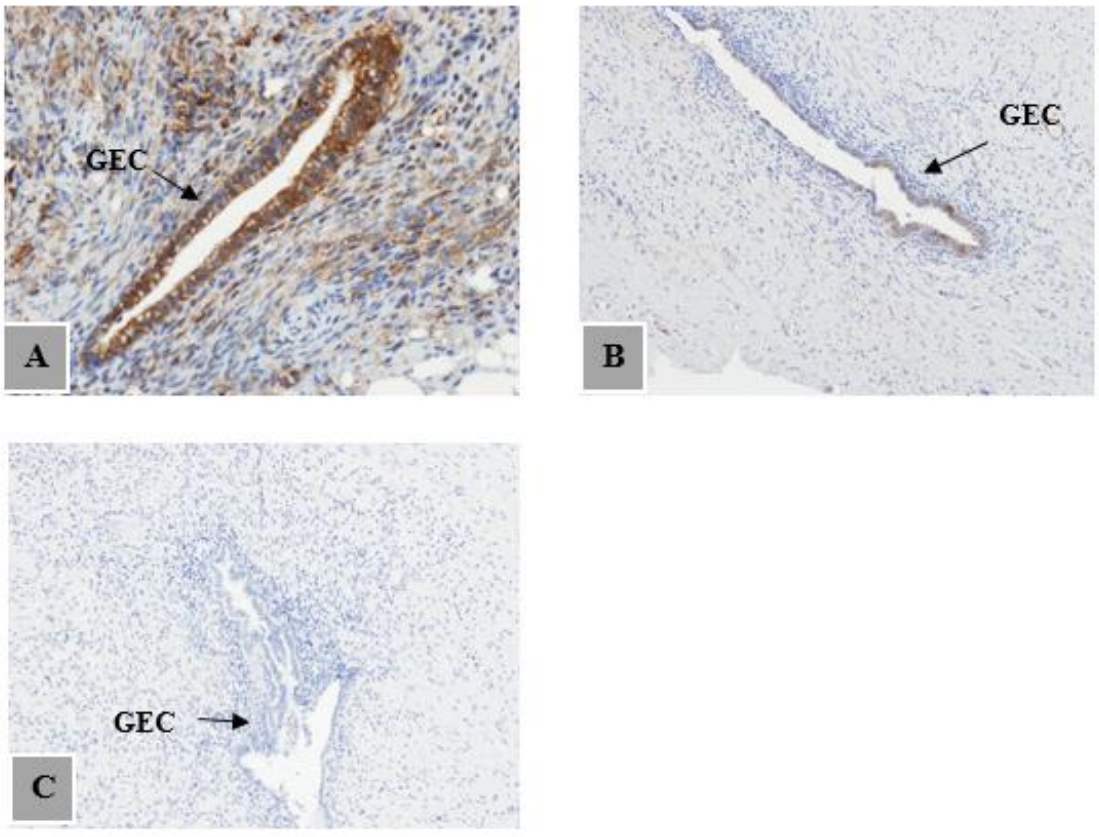

Figure 5.1: The difference in PAI-1 immuno-intensity in glandular epithelial cells (GECs) between the Study Group and the Control Group.

An increase of PAI-1 immuno-intensity in GECs was observed in the Control Group **(A)** (lower nerve bundles density) compared to the Study Group **(B)** (higher nerve bundles density). **(C)** Sections without primary antibody as a blank control (20×).

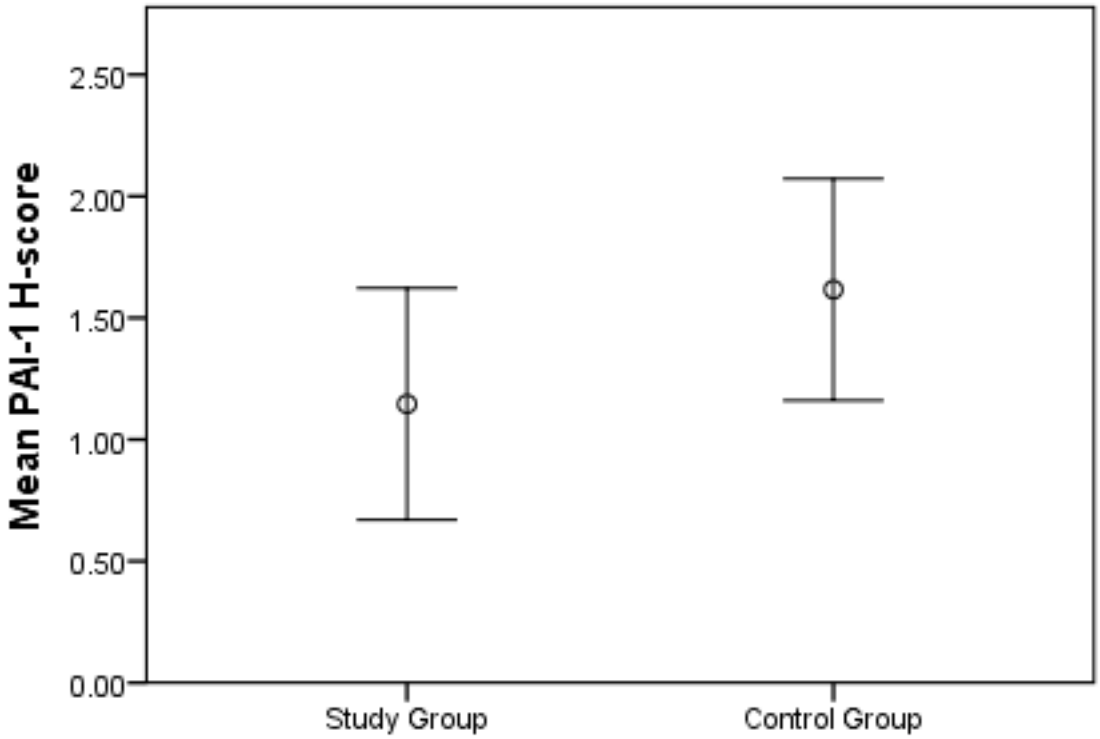

Figure 5.2: Analysis of PAI-1 immunoreactivity in the Study Group and the Control Group using the Histoscore (H-score).

Mean H scores (± standard deviation [SD]) for the staining intensity of PAI-1 expression in both the Study Group and the Control Group for GECs. There was a trend towards a decrease of PAI-1 immuno-intensity in GECs in the Study Group (higher nerve bundle density) compared to the Control Group (lower nerve bundle density). However, this difference was not significant by Histoscore analysis ($p = 0.068$).

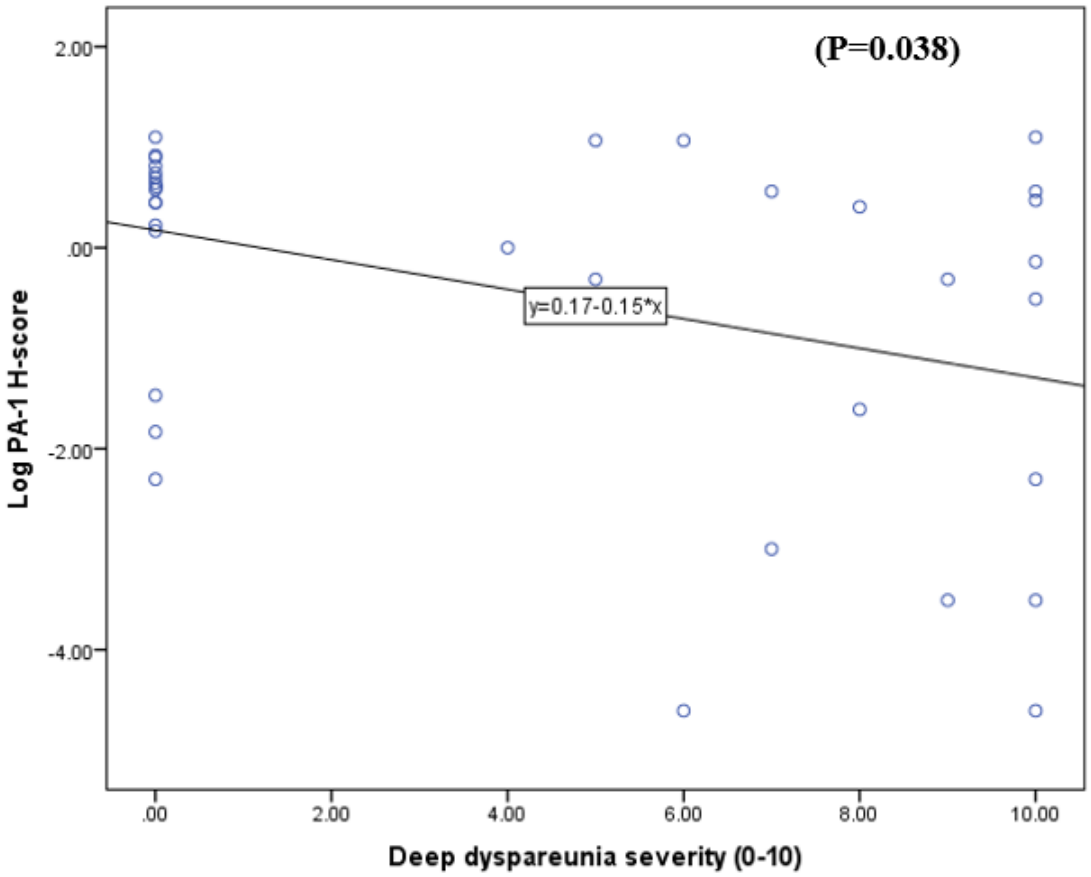

Figure 5.3: Log PAI-1 H-score and severity of deep dyspareunia.

Scatterplot with a linear fit line showing the correlation between PAI-1 H-score and deep dyspareunia severity (Spearman= -0.352, n=35, p = 0.038).

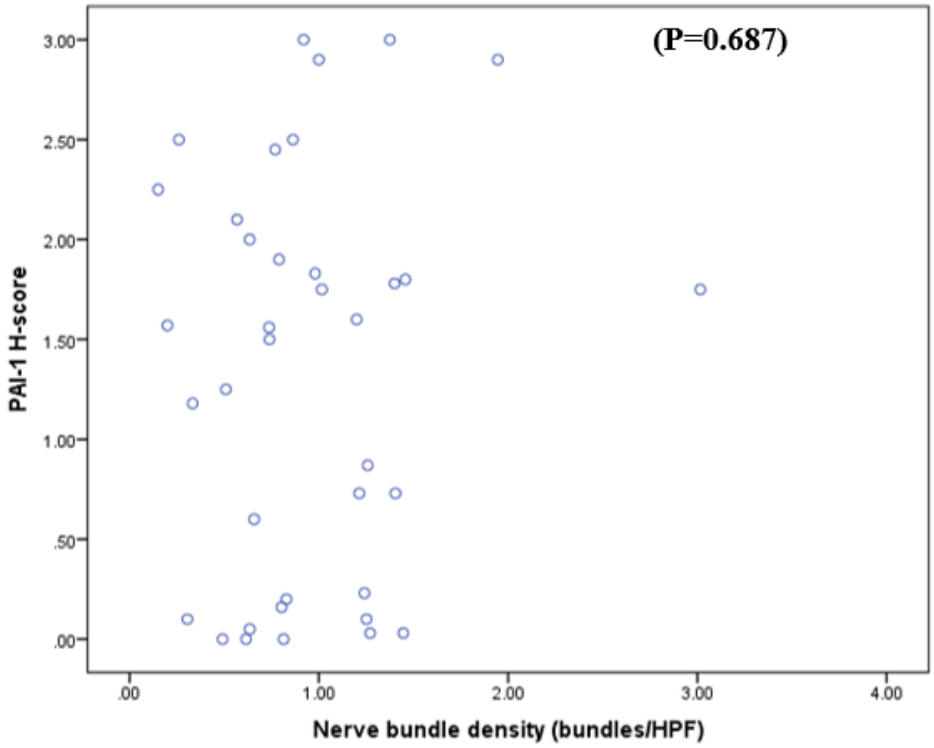

Figure 5.4: PAI-1 H-score and nerve bundle density.

There was no correlation between PAI-1 H-score and nerve bundle density ($r = 0.069$, $p = 0.687$).

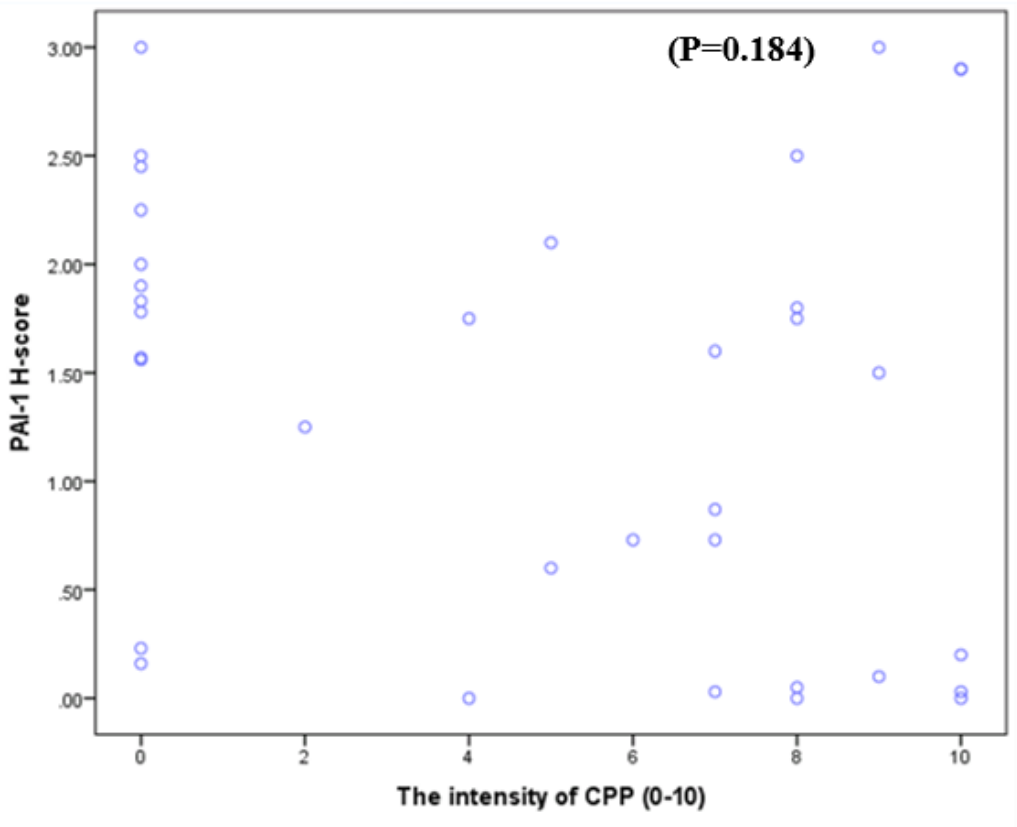

Figure 5.5: PAI-1 H-score and intensity of chronic pelvic pain.

There was no correlation between PAI-1 H-score and intensity of chronic pelvic pain ($r = -0.230$, $p = 0.184$).

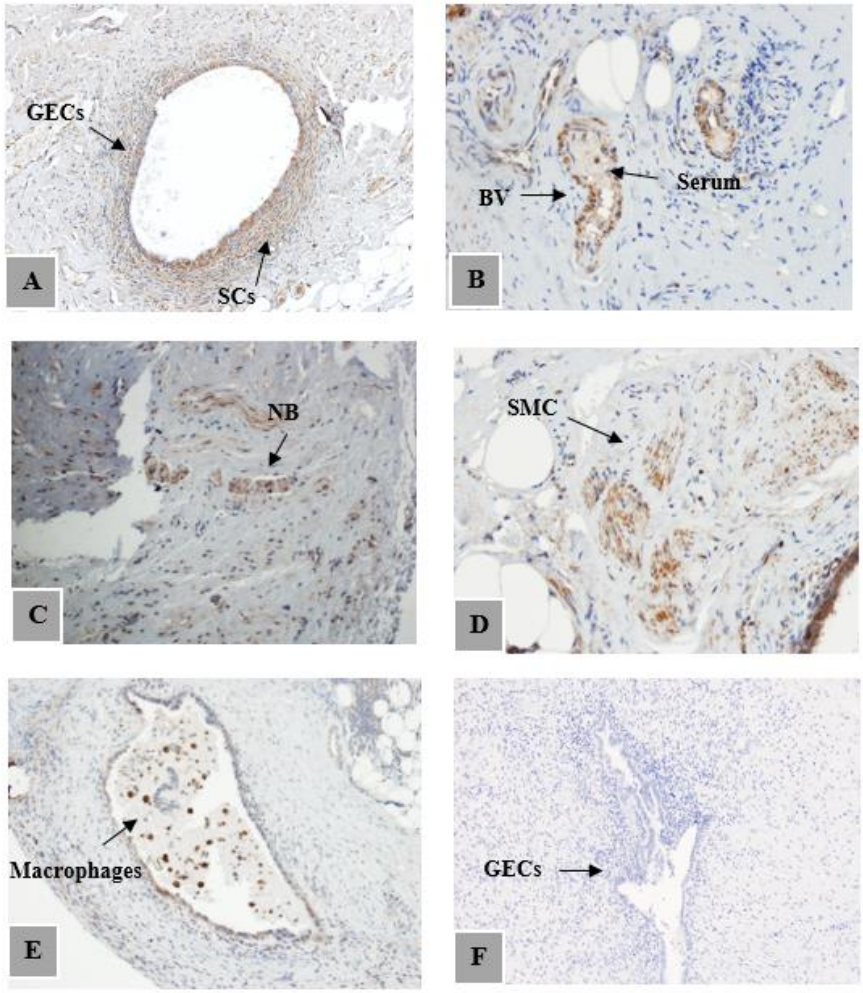

Figure 5.6: The localization of PAI-1 protein in endometriotic and surrounding tissues from women with endometriosis.

All sections were immunostained by the monoclonal PAI-1 antibody at dilutions 1:25. Positive PAI-1 staining was seen in endometriosis glandular epithelial cells (GECs) and stroma cells (SCs) **(A)**. Also PAI-1 stained positive in the endothelium of blood vessels (BV) and serum **(B)**, in nerve bundles (NB) **(C)**, in smooth muscle cells (SMCs) **(D)**, and also in macrophages **(E). (F)** Sections without primary antibody as a blank control (20×).

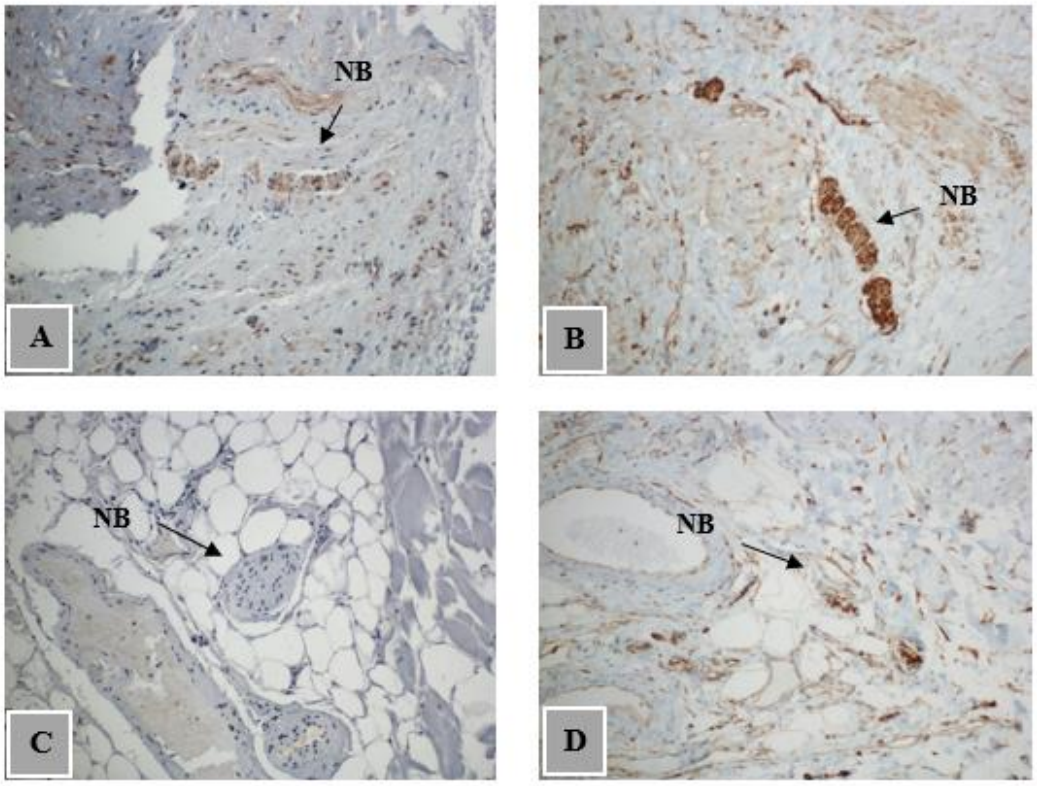

Figure 5.7: PAI-1 expression in nerve bundles (NB) in the Study Group and the Control Group.

Nerve bundles from women with endometriosis showed variable PAI-1 immunoreactivity. PAI-1 stained positive in 13 cases of the Control Group **(A)** and 13 cases of the Study Group **(C). (B)** and **(D)** are the pan-neuronal marker (PGP9.5) stained sections from our previously published paper [73] and these sections were used to identify nerve bundles (20×).

5.4 Discussion

Endometriosis is a common, estrogen-dependent, chronic gynecological disorder associated with pelvic pain, such as deep dyspareunia, and infertility. In particular, deep dyspareunia is present in half of women with endometriosis [43] [44]. The devastating consequences of endometriosis-associated deep dyspareunia affecting women's health are well documented. Sexual activity and self-esteem are reduced due to endometriosis-associated deep dyspareunia [44].

Plasminogen activator inhibitor-1(PAI-1), or serpin E1, is a serine protease inhibitor (serpin) encoded by the human SERPINE1 gene. PAI-1 is a major inhibitor of fibrinolysis, a process that prevents blood clots from growing and becoming problematic. Specifically, Wagner et al. showed that PAI-1 binds to fibrin [164] thus PAI-1 activity may result in depressed fibrinolytic activity.

Hypofibrinolysis is suggested to allow persistence of the local fibrin matrix that could act as a scaffold for nerve fiber formation [128]. Also, there is some empirical evidence that increased PAI-1 expression helps to promote neuron survival, for example through a reduction in apoptosis [126]. Based on this, I hypothesized that PAI-1 expression would be increased in women with endometriosis and deep dyspareunia, compared to women with endometriosis and no deep dyspareunia.

Interestingly, in contrast to our hypothesis, we found that decreased PAI-1 expression in endometriosis glandular epithelium was significantly correlated with increased severity of deep

dyspareunia ($r = -0.352$, $p = 0.038$) in the total cohort. When women were classified into a Study Group (deep dyspareunia) and a Control Group (no deep dyspareunia), there was also a trend towards decreased PAI-1 expression in the Study Group, although not statistically significant. Moreover, PAI-1 expression was not correlated with nerve bundle density.

Our findings raise the possibility that PAI-1 expression may actually be decreased in women with endometriosis deep dyspareunia, and this association is independent of nerve bundle density. Perhaps, decreased PAI-1 promotes a hypocoagulable environment and increased bleeding in the endometriotic lesions, which is one of the longstanding postulated mechanisms for how endometriosis cause pain [165]. Another explanation for this finding is that reduced PAI-1 levels may lead to increased fibrinolysis and an associated increased vaginal bleeding during menstruation, which may also provoke more pelvic pain in general including during intercourse.

This finding is consistent with published data that actually showed a negative correlation between PAI-1 levels and sexual dysfunction [166]. Through a questionnaire (Female Sexual Function Index [FSFI]), Veronelli et al. evaluated FSFI score, endocrine and metabolic parameters, including PAI-1, in diabetic, in obese, and in hypothyroid women vs. healthy women. It is important to know that there was no data about whether these women had endometriosis or not. They reported that PAI-1 was inversely correlated with FSFI score ($p < 0.05$) and negatively predicted reduced FSFI score ($F = -4.214$, $p = 0.025$) (i.e. lower PAI-1 was associated with higher FSFI or worse sexual function).

As mentioned before, we observed positive PAI-1 staining in both endometriotic glandular epithelial cells and stroma cells in both the Study Group and the Control Group. PAI-1 expression (mRNA levels) was previously reported to be expressed in endometriotic stroma [167]. Also, in a recent study, it was shown that endometriotic cells secreted more PAI-1 than the endometrial cells, and that stromal cells (endometrial and endometriotic) secreted considerably more PAI-1 compared to epithelial cells [107]. Moreover, our finding of PAI-1 expression in the endothelium of blood vessels, nerve bundles, smooth muscle cells, macrophages is consistent with previously published data [136] [137] [138] [139].

A strength of our study is the use of a validated antibody (see Chapter 2 for validation method). In addition, in order to avoid bias, a blinding score system was employed for this study to estimate PAI-1 expression in endometriotic tissues from the Study Group and the Control Group. Also, this Study Group and Control Group were highly phenotyped, combining physical exam and surgical and histological findings.

One limitation of the study is that it was conducted on retrospective cases. Also, only one representative slide per case was examined which may not the reliable representation of PAI-1 levels in these women. In this study, we utilized only PAI-1 antibody, and so we do not have data on whether t-PA or u-PA may correlate positively with deep dyspareunia as those two activators are well known to be inhibited by PAI-1.

Chapter 6: Conclusion and Future Directions

The role of PAI-1 in the development of different diseases, including deep vein thrombosis [168], myocardial infarction [169], fibrotic disorders [170], metabolic disorders [171], and cancer [172], has been well recognized. In the reproductive system, PAI-1 has been reported to involve the development of endometriosis [141] [144] [143]. Moreover increased PAI-1 expression has some roles in neuron survival [126], there was a negative correlation between PAI-1 levels and sexual dysfunction [166]. For these reasons, the experiments described in this thesis aimed to not only establish a connection between this molecular marker and a chronic reproductive disease, endometriosis, but also to investigate any correlation between PAI-1 and clinical symptoms such as deep dyspareunia.

First, utilizing our validated antibody we have demonstrated that PAI-1 protein expression was immunolocalized in endometriotic glandular epithelial cells and stroma cells. Second, our results demonstrated higher expression of PAI-1 in a deep infiltrating endometriosis (DIE) group compared to other groups. In particular, PAI-1 was significantly higher in the DIE group compared to the superficial endometriosis group and eutopic endometrium group where there is no invasion process. Third, using our previously published cohort [74], we found a decrease of PAI-1 immuno-intensity in GECs in women with cul-de-sac endometriosis and more severe deep dyspareunia.

Regarding neurogenesis in endometriosis, our lab recently completed a study to investigate the expression of nerve growth factor (NGF) and its receptor in endometriotic tissues from the same two groups, i.e. women with cul-de-sac endometriosis with or without deep dyspareunia. NGF has

been reported to induce nerve growth in different tissues [173]. We found that higher NGF and receptor expression was associated with the Study Group with deep dyspareunia (Peng et al., submitted).

A stronger association was observed between PAI-1 expression and DIE, than between PAI-1 expression and the symptom of deep dyspareunia. DIE is defined as an endometriotic lesion penetrating to a depth of 5 mm or more [25], where the intensity of pain was found to be proportional to the depth to which the lesions penetrate [25]. DIE causes unique symptoms such as those involving the urinary tract (dysuria, urinary infection, sometimes hematuria) or the bowel (rectal tenesmus and sometimes hematochezia). The management of DIE, whether medically or surgically, can be challenging [174]. Thus, understanding pathophysiology of DIE is important, to provide novel treatment targets.

PAI-1 could be a possible therapeutic target in deep endometriosis. Recently, it was found that treatment with the small molecule PAI-1 inhibitor TM5275 effectively blocked cell proliferation of ovarian cancer cells that highly express PAI-1 [175]. For example, small molecule inhibitors of PAI-1 (TM5275 and TM5441) were also shown to decrease cell viability in several human cancer cell lines [176]. It was suggested that PAI-1 inhibition promotes cell cycle arrest and apoptosis in ovarian cancer and that PAI-1 inhibitors potentially represent a novel class of antitumor agents.

For future research, I plan the following. First, sample sizes of deep infiltrating endometriosis and other subtypes of endometriosis will be increased and cases without endometriosis will be included as controls, to confirm the findings in this thesis. Second, I will explore whether overexpression or knockdown of PAI-1 and uPA alter the invasive ability of endometriosis cell lines or primary cultures. If so, I will study the mechanism by which these factors can promote cell invasion in endometriosis, for example by upregulating expression of integrin α5 and VEGF [177]. Third, I will utilize small molecule inhibitors for both factors to determine whether they can reduce endometriosis cell invasion in vitro.

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