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An investigation of nerve fibres in the endometrial functional layer and peritoneal lesions of adolescents with endometriosis

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A thesis submitted in fulfilment of the requirements for the degree of Master of Philosophy in Medicine

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ABSTRACT

Background

Endometriosis is an enigmatic disease of uncertain aetiology and pathogenesis, characterised by the presence of endometrial-like tissue in areas outside the uterine cavity. It is a chronic disease associated with pelvic pain and subfertility. Endometriosis in adolescents presents a particular challenge of differential diagnosis and choice of treatment. The disease often begins in adolescence, but is most often recognised after years of dysmenorrhea. Some data suggests that two thirds of adult women with endometriosis report the onset of their symptoms before the age of 20. Furthermore, a recent multi-centre study conducted on behalf of the World Endometriosis Research Foundation Global Study of Women’s Health consortium showed that the diagnostic delay is an average of 6.7 years for affected women. The combination of variable clinical presentation that is difficult to distinguish from primary dysmenorrhoea and the shortage of non-surgical methods for diagnosis has led to this delay. The pain from the delay and misdiagnosis has a debilitating effect on the quality of life for these women. Facilitating earlier diagnosis and intervention for patients with endometriosis has potential to prevent disease progression, psychological pain and preserve future fertility. More recently, studies have emerged with promises of markers for endometriosis with the prospect of using this method as a less invasive definitive diagnosis for endometriosis. However, it is important to recognise that this progression is limited to adult women with less focus being the adolescent group.

Previous studies have demonstrated that sympathetic, parasympathetic and sensory nerve fibres innervate peritoneal lesions of endometriosis. Following these studies and despite being
controversial, nerve fibres have also been detected in the functional layer of women with endometriosis. These studies insinuate a role of endometrial nerve fibres for pathophysiology of endometriosis-associated pelvic pain. However, there currently exists no data that discusses the innervation of peritoneal lesions or eutopic endometrium of the adolescent.

**Aims**

This pilot study aimed to characterise and study the presence of nerve fibres, detected with neuronal markers: Protein gene product 9.5 (PGP9.5), nerve growth factor (NGF) and its high affinity growth factor receptor tyrosine kinase A (Trk-A) in the functional layer of the endometrium and peritoneal lesions of adolescents with laparoscopically visualised and histologically confirmed endometriosis. We sought to examine how nerve fibre density correlated with clinical symptoms, menstrual stage and severity of disease in adolescents with endometriosis.

**Methods**

The study included the identification of endometrial biopsies samples taken at dilatation of cervix and curettage (D&C) of the endometrium from adolescent females who underwent investigation for pelvic pain at the Women’s Hospital in Melbourne between 2004 and 2009. Clinical records were available providing the nature of their symptoms, the extent and stage of endometriosis. Endometrial biopsies taken at the time of laparoscopy were fixed into paraffin blocks in preparation for immuno-histochemical staining procedures. The embedded blocks were cut into 4μm sections and immuno-histochemically stained for antibodies that were previously shown to be markers of endometriosis in adult women (≥ 21 years old). Tissue morphology, density of nerve fibres and nerve growth factor levels were analysed by microscopy. A
relationship analysis was performed between nerve fibre densities, NGF and Trk-A levels, clinical symptoms, severity of disease and menstrual cycle.

**Results**

This study showed that 33.33% (18 of 54) of adolescent endometriotic eutopic endometrial samples expressed nerve fibres detected by PGP 9.5, with NGF expression in 68.52% (37 of 54) and Trk-A expression in 42.59% (23 of 54). It also demonstrated higher nerve fibre density expression by PGP 9.5 in adolescent endometriotic peritoneal lesions with 70% (21 of 30) compared to the eutopic endometrial samples. However, the other two markers had lower expression with NGF levels of 50% (15 of 30) and Trk-A levels of 33.33% (10 of 30) in these adolescent endometriotic peritoneal lesion samples when also compared to the eutopic endometrial tissue samples.

Nerve fibre density in endometrial tissue samples had a mean of 57.70mm$^2$, with no pattern of distribution between stromal and peristromal regions. Nerve fibre density in lesion samples showed a higher density in the peristroma (median nerve fibre density of 90.11mm$^2$) than those seen in the stroma (median nerve fibre density of 4.0mm$^2$). NGF and Trk-A expression in endometrial samples showed positive correlations between glands and stroma with normal distribution, suggesting that when the expression of NGF and Trk-A was seen in the glands it was also seen in the stroma. However the intensity levels of these markers were higher in the glands when compared to the stroma with p values of 0.010 for NGF levels and <0.001 for TrkA levels. NGF and Trk-A expression in peritoneal lesions showed similar correlations, with positive correlations between glandular and stromal NGF and Trk-A and the expression was also higher in the glandular region than the stroma (p = 0.028 for NGF and <0.001 for Trk-A).
Interestingly, our study also showed a statistically significant correlation between stroma nerve fibre density and stroma NGF expression ($p>0.05$).

No correlations of nerve fibre densities/expression were found with medical treatment, severity of disease, menstrual stage and age.

**Conclusion**

The findings of this study suggest that nerve fibres known to contribute to pelvic pain in older women seen in the functional layer of the endometrium and peritoneal lesions are also detected in adolescents with endometriosis. Our study proposes that increased nerve fibres in the stroma of adolescent endometriotic lesion may be implicated in mechanism of pain generation in endometriosis. Increased NGF and Trk-A expression in the glandular epithelium of endometrial and peritoneal lesions suggest that nerve fibres may be encouraged to grow/progress into the core area of endometriotic lesions. Although we did not find a correlation between nerve fibre density/neuronal intensity with severity of disease, menstrual stage and age, we did find that oral contraceptives or combined hormonal therapy did not make a difference in nerve fibre expression in these adolescent endometriotic tissues. Further prospective studies with greater sample size and a control group are needed to shed light on the mechanisms of pain generation in endometriosis.
DECLARATION

I, Amy Thi Nguyen, declare that the contents of this thesis consist of original work carried out by the author unless otherwise stated and duly acknowledged. To the best of my knowledge no part of this thesis has been submitted in whole or in part for any other degree.

Amy T. Nguyen
September 2014
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LIST OF ABBREVIATIONS

PGP 9.5 – Protein gene product 9.5
NGF – Nerve growth factor
Trk- A –Tyrosine kinase A
D&C – Dilatation and curettage
USA – United States of America
UK – United Kingdom
OC – Oral Contraceptives
NSAIDs – Non-steroidal anti-inflammatory drugs
HCUP - Healthcare Cost and Utilisation Project
MRI - Magnetic Resonance Imaging
GnRH - Gonadotropin-Releasing Hormone
FSH - Follicular Stimulation Hormone
α-SMA - α-smooth muscle actin
ASRM - American Society of Reproductive Medicine
CHT - Combined Hormonal Therapy
IUD - Intrauterine system
CNS - Central Nervous System
PNS - Peripheral Nervous System
DRG - Dorsal Root Ganglia
COX – Cyclooxygenase
NF – Neurofilament
VIP – Vasointestinal peptide
NPY – Neuropeptide Y
SP – Substance P
CGRP – Calcitonin Gene-Related Peptide
VEGF - Vascular Endothelial Growth Factor
EPCs - Endothelial Progenitor Cells
IL-8 - Interleukin 8
NK – Natural Killer cell

17β HSD2 - 17β Hydroxysteroid Dehydrogenase Type 2
E₁ – Oestrone
E₂ – Oestradiol
PGE₂ – Prostaglandin
PGF₂α – Prostaglandin F₂α
H & E - Haematoxylin and Eosin
DHM - Douglas Hanly Moir Pathology
IHC – Immunohistochemistry
Ab – Antibody
Ag – Antigen
Fab - Fragment Antigen Binding
Fc - Fragment Crystalline
COO – Carboxyl
NH₂ – Amino Terminal
DAKO – Dakocyтомation
DAB - 3-Diaminobenzidine Tetrahydrochloride
SD – Standard Deviation
COCP – Combined Oral Contraceptive Pill
Depo – Depo-Provera
IQR - Interquartile Range
n.f. – Nerve fibre
GAP-43 - Growth Associated Protein -43
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CHAPTER 1 – ENDOMETRIOSIS IN THE ADOLESCENT

1.1 Definition

Endometriosis is an enigmatic disease of uncertain aetiology and pathogenesis characterised by the presence of endometrial glands and stroma histologically similar, but not identical to the inner lining of the uterus (the endometrium) and at sites outside the uterus (Hudelist et al. 2009). The inner menstruating layer of the uterus in women with endometriosis is termed the eutopic endometrium, whereas the abnormal tissues outside the uterus are termed “endometriotic lesions”.

1.2 General Perspectives

Endometriosis is a chronic oestrogen-dependent gynaecological disease affecting millions of women worldwide (Giudice & Kao 2004). Despite the fact that endometriosis has been studied for nearly a century, it remains an enigmatic condition with disputed aetiology amongst clinicians and researchers with no cure (Rizk & Abdalla 2003). Endometriosis is frequently associated with debilitating pelvic pain i.e. pain during menstruation (dysmenorrhoea), sexual intercourse (deep dyspareunia), non-cyclic chronic pain with ovulation or bowel movements, and infertility (Goldstein et al. 1980). However, the relationship between pain and endometriosis still remains unclear. A number of studies suggested a link between nerve fibres and the pathophysiology of endometriosis and endometriosis related pain (Asante & Taylor 2011; Fraser et al. 2011; Tomita & Mah 2014), with the prospect of using an increased expression of nerve fibres in endometrial biopsies as a definitive diagnosis for endometriosis (Al-Jefout, Dezarnaulds, et al. 2009).
This disorder is most commonly diagnosed in women of reproductive age, although the time to actual diagnosis can be quite delayed with a mean of 11.7 years in the United States of America (USA) and 8.0 years in the United Kingdom (UK) (Perloe 2007). It has become progressively evident that this impediment is due to the fact that the extent of endometriosis is extremely variable and does not often correlate with the frequency and the severity of symptoms or with long term prognosis in terms of recurrence and contraception (Giudice & Kao 2004).

For many women, endometriosis imposes a substantial burden in terms of well-being, personal relationships, time off work, and need for surgery and expensive therapies. In addition, findings have suggested that these women also have an increased risk of ovarian, breast and skin cancer, as well as autoimmune diseases (Swiersz 2002). Therefore, it is not only essential for greater awareness of the possible diagnosis of endometriosis in patients with pelvic pain, but it is becoming particularly important to do so in adolescent girls for earlier and accurate therapy.

1.3 Endometriosis in Adolescents

Endometriosis in the adolescent population has been particularly challenging in the field of gynaecology with differential diagnosis for pelvic pain and choice of treatment. The presentation of endometriosis in adolescents is variable, and it is often difficult to distinguish from primary dysmenorrhoea (Yang et al. 2012). There is limited scientific literature on endometriosis specific to the adolescent with data that is retrospective and descriptive in nature. It has been speculated that this disease has different pathophysiology in adolescents, however
there is little epidemiologic or molecular data that exist to either support or refute this concept (Shah & Missmer 2011). The literature has not confirmed that intervention in the adolescent prevents long-term sequelae. Therefore further studies to identify risk factors and treatment outcomes are needed for further understanding of endometriosis in the adolescent population (Laufer 2008).

1.4 Epidemiology

There is currently no epidemiological data that is available for the true incidence or prevalence of endometriosis in adolescents, although data from the Endometriosis Association indicate that 66% of adult women with endometriosis reported the symptoms starting before the age of 20 (American College of Obstetricians and Gynaecologists 2005).

1.4.1 Extent

The extent of endometriosis is extremely inconsistent due to the variability in presentation and it does not often correlate with the severity of symptoms. Although it is often associated with severe pain and infertility, it can sometimes be asymptomatic. The complications in the diagnosis of endometriosis have led to long delays from onset of symptoms to the actual establishment of the diagnosis itself. Although endometriosis is popularly presented in women of reproductive age, there have been documented symptomatic cases prior to menarche in girls who have some breast development and others soon after menarche (Laufer 2000; Goldstein et al. 1979; Yamamoto et al. 1997).
There is evidence that some adolescents have a genetic predisposition to developing endometriosis. In one particular study involving patients with histologically confirmed endometriosis, first degree female relatives of affected patients were significantly more likely to have been diagnosed with endometriosis compared to relatives (Simpson et al. 1980).

1.4.2 Prevalence

The true prevalence of endometriosis is not clear as there have been long term difficulties in its diagnosis. The estimates that have been reported in the literature are wide and vary depending upon the population studied (symptomatic or asymptomatic) and the method of diagnosis (clinical vs. surgical). Additionally, further complications of these estimates are caused by a small population of women who are symptomatic but do not present themselves medically. Consequently, this cohort goes undetected in the population. Despite these impediments, several studies have suggested that the prevalence of endometriosis ranges from 30% to 80% in women with pelvic pain and 9% to 50% in women undergoing a laparoscopy for evaluation of infertility (Missmer et al. 2004; ASRM: American Society for Reproductive Medicine 2012).

Common among adolescents as well as adults, endometriosis has been observed in females as young as the age of 10 (Gould 2003). Published incidence rates vary among adolescents with chronic pelvic pain with reported rates of 25% to 38% (Vercellini et al. 1989; Kontoravdis et al. 1999) and 47% in those with chronic pelvic pain that have undergone
laparoscopy (Goldstein et al. 1980). Of those adolescents with pelvic pain uncontrolled by medical management with oral contraceptives (OCs) and non-steroidal anti-inflammatory drugs (NSAIDs), the incidence has been shown to be as high as 60% to 70% at time of laparoscopy (Laufer 2012; Laufer et al. 1997; Reese et al. 1996). Although these rates vary for chronic pelvic pain, one study estimated an incidence of 12% among girls between the age of 11-13 years (Propst & Laufer 1999). In addition, another study from Boston Children’s Hospital found that teenagers typically presented with early stages of endometriosis, with 77% presenting with Stage 1 and 23% at stage 2 (Propst & Laufer 1999).

1.4.3 Burden of Disease

Endometriosis has been linked to other burdens aside from the physical pain. Studies have concluded that it impairs health related quality of life, specifically in areas related to physical, psychological, and social function. In addition, with its wide range of clinical presentation, endometriosis has been difficult to diagnose with high health care costs.

There are currently no studies that quantify the economic impact of endometriosis among adolescents. However, the quality of life impact of endometriosis in this population has been documented. Adolescents have been noted to experience depression, fear, or anxiety which may have resulted in increased use of medical resources and concomitant costs. Similarly to adult women with work, adolescent endometriosis can affect school attendance (Laufer et al. 1997). As mentioned earlier, pelvic pain and dysmenorrhoea, which are common symptoms of endometriosis, affect 45%-70% of adolescents. One survey of 2699 menarchal adolescent girls showed that 25% of all excessive school
absences for these girls were due to dysmenorrhea or pelvic pain (Klein & Litt 1981; Gao et al. 2006). In particular, 50% of girls with severe dysmenorrhea or pelvic pain reported school absences due to their cramps (Klein & Litt 1981). This survey was conducted over 30 years ago, and no recent or similar published data has been found in the literature.

1.4.4 Cost

Endometriosis is a very costly public health problem as it has high rates of hospital admissions, surgical procedures, and incidences of comorbid conditions (Gao et al. 2006). In effect, studies have shown that the yearly total (direct plus indirect) cost of endometriosis has been estimated at €30 billion in Europe and $22 billion in the USA, with direct costs still increasing steadily (Simoens et al. 2007; Gao et al. 2006).

The study by Zhao et al. (1998) studied the entire hospitalisation as compared to many of the other studies that focus on the procedural costs. This study included hospitalisation rates, frequency of inpatient stay, and related costs of endometriosis using the Healthcare Cost and Utilisation Project (HCUP). They found that the mean inpatient charges per admission for endometriosis were $6,597 in 1991 and $7,449 in 1992. The mean length of stay for women with endometriosis as a principal diagnosis was 3.8 and 3.5 days in 1991 and 1992, respectively.

Social, indirect, and intangible costs also contribute to the overall economic consequences of endometriosis. These costs include, but not limited to, loss in work productivity, loss of income, social withdrawal, and psychological disorders such as depression (Gao et al. 2006). Although endometriosis may be an economic burden to the
health care systems, very few studies exist that quantify the indirect impact. In a multi-centre study across ten countries, the impact of endometriosis on quality of life and work productivity was observed across ten different ethnicities (Nnoaham et al. 2011). In total, 1,486 of 1,669 eligible women agreed to participate. They found that affected women reported a greater number of absences from a duty or obligation and a higher number presented to work while sick when compared with symptomatic control women. Overall work productivity loss was 10.8 hours/week compared to 8.4 hours/week which rose with increasing disease severity (Nnoaham et al. 2011).

Absenteism-related costs ranged from US$1/ week in Nigeria to US$250/week in the United States. Although reduced effectiveness at work is less frequently assessed and recorded than work absence, it accounted for nearly 60% of total work productivity loss. The annual costs per employed woman of endometriosis associated work productivity loss varies from US$209 in Nigeria to US$23,712 in Italy (Nnoaham et al. 2011).

1.5 Anatomy of the Uterus

As mentioned earlier, endometriosis is a disorder in which tissue that normally lines the inside of the uterus (the endometrium) grows outside the uterus. For a better understanding of the disease, a basic knowledge of the anatomy is needed.

1.5.1 Uterus

Endometriosis is a condition in which the endometrium (the tissue that normally lines the uterus), grows outside the uterus. The female reproductive tract is a system of interconnected passages that encompasses the internal cavities of the uterus, uterine
tubes, and vagina (Germann & Stanfield 2002). The centrepiece of this coordinated system is the hollow muscular organ, held in the middle of the pelvic cavity known as the uterus. The uterus is held in place by several condensations of endopelvic fascia called ligaments and is covered by a sheet like fold of peritoneum known as the broad ligament (Burlev et al. 2005). The uterus comprises of four main regions shown in Figure 1.1, the fundus, the body of the uterus, the isthmus, and the cervix.

![Figure 1.1 Posterior view of the uterus and associated structures](http://www.britannica.com)

The curved, broad area of the uterus connected to the uterine tubes is called the fundus. The body is the main part of the uterus that starts directly below the level of the fallopian tubes and continues downward until the uterine walls and cavity start to narrow. The
isthmus is the lower, narrow neck region and the lowest section is the cervix, which extends downward from the isthmus until it protrudes into the vagina (Martini et al. 2009). The dimensions of the uterus are highly variable but normally ranges from 6-8cms in length and its walls usually have a thickness of 2-3cms. In women of reproductive age, the uterus is much wider at the fundus in comparison to the uterus of a premenarche female child, which is much smaller and narrower through the entire body. During menopause, the uterus becomes paler, less muscular and regresses in size (Rocha et al., 2012).

To accommodate for these roles the uterine wall is strong and flexible, comprising of three layers (Figure 1.1). The perimetrium, the outermost layer beneath the layer of epithelial cells and connective tissue, the muscular myometrium and an inner glandular layer where important biological functions occur called the endometrium. Initially, the reproductive function of the uterus is to accept the fertilised egg from the uterine tubes. Thereafter it houses and nourishes the developing foetus until childbirth. The uterus is also essential in sexual response as it directs blood flow to the pelvis and to the external genitalia including the ovaries, vagina, labia and clitoris.

1.5.2 The Myometrium and Endometrium

The myometrium is the thickest portion of the uterine wall, forming nearly 90 percent of the mass of the uterus (Martini et al. 2009). The smooth muscle in the myometrium is arranged into longitudinal, circular and oblique layers. Contractions of these layers provide the force needed to expel the foetus out of the uterus and into the vagina. The endometrium contributes to approximately 10 percent of the mass of the uterus. It consists
of the functional endometrium and the basal endometrium. There are a vast number of uterine glands that open onto the endometrial surface, extending deep into the lamina propria, nearly reaching the myometrium.

The endometrium varies greatly depending on the phase of the menstrual cycle. Under the influence of hormones, the uterine glands, blood vessels, and endothelium change with the various phases. The uterine endometrial cycle (menstrual cycle) can be divided into three phases: the proliferative/follicular phase, the luteal/secretory phase and the menstrual phase. The stages are coordinated by a complex and intricate neuro-endocrine mechanism (Jones & Lopez 2006). This mechanism controls the fluctuation levels of oestradiol and progesterone secreted by the ovary. It is these fluctuations in the hormones that cause endometrial growth and menstruation and it also opens a window for implantation in the immediate postovulatory period, should fertilisation occur.

1.5.3 The Menstrual Cycle

Menstruation generally occurs every 22 to 35 days, with cycles varying at both extremes of the reproductive group. For women with 28-29 day cycle lengths, the menstrual cycle may be divided into the three general phases. Figure 1.2 shows that during these three phases, there are changes in the cyclic endometrium thickness, vasculature, and follicle depending on changes in hormones (Martini et al. 2009). The changes in endometrium thickness can be detected using magnetic resonance imaging (MRI) (Hricak et al. 1983).
Figure 1.2: The menstrual cycle: the changes in follicular growth, body temperature, endometrial thickening and hormonal regulation during the menstrual cycle

(Martini et al., 2009).
During the beginning of the menstrual cycle; the menstrual phase corresponds to the period of menstruation (Figure 1.2). It is triggered in response to a decline in plasma oestrogen and progesterone due to degeneration of corpus luteum (Maki & Resnick 2001). Menstruation last for three to five days. During the onset, blood vessels in the outermost layer of the endometrium begin to constrict, reducing blood flow to the tissue. As a result, these tissues die and begin separating from the underlying endometrial tissues. These dead tissues gradually shed from the endometrial surface proliferating blood vessels causing bleeding. The mixture of blood and tissue migrate from the uterus into the vagina, and menstrual flow occurs (Germann & Stanfield 2002).

The low level of oestrogen and progesterone stimulates Gonadotropin-releasing hormone (GnRH) resulting in secretion of follicle-stimulating hormone (FSH) from anterior pituitary. The follicular phase itself typically lasts from the end of menstruation to Day 14, marking ovulation. The early stages of this cycle are characterised by low serum concentrations of both oestradiol, and progesterone (Maki & Resnick 2001). Oestradiol peaks in the pre-ovulatory surge immediately prior to ovulation in preparation for possible pregnancy, while progesterone remains low. The remaining endometrial tissues from the menstrual phase begin to grow, and the smooth muscle in the underlying myometrium thickens. Meanwhile, the increasing levels of oestrogen induce the proliferation of the functionalis from stem cells of the basalis, endometrial glands, and stromal connective tissue as seen in the diagram of the uterine wall shown in the bottom of Figure 1.2. The endometrium can measure 1-3mm during this phase.
Following the proliferative phase is the secretory or luteal phase, which commonly extends from Days 14 to 28 and is distinguished by high concentrations of both oestrogen and progesterone caused by the corpus luteum. At the beginning of the luteal phase, progesterone further induces the endometrial glands and begin secreting fluids rich in glycogen (Mueller et al. 2000). The blood supply of the endometrium becomes enriched as arteries branch and is between 3-5 mm in thickness. In the absence of pregnancy, corresponding with the end of the secretory phase, the corpus luteum degenerates, causing plasma oestrogen and progesterone to fall which, creates a withdrawal in their growth influences on the endometrium and triggers the previously described events of menstruation. If fertilisation and implantation have occurred, the corpus luteum does not degenerate and oestrogen and progesterone levels remain elevated to support pregnancy.

1.5.3 The Peritoneum

The peritoneum is the serous membrane composed of a layer of mesothelium supported by a thin layer of connective tissue (Figure 1.1). It functions as a support for abdominal organs and a conduit for their blood and lymphatic vessels and nerves. The body of the uterus is mostly covered with peritoneum. Anteriorly, the peritoneum is only loosely adherent allowing for bladder distension and posteriorly, the peritoneum continues downwards to cover the upper quarter of the posterior wall of the vagina (Ellis 2011). The uterine artery arises from the internal iliac artery, runs in the base of the round ligament and immediately crosses above, passing superficially to the ureter to reach the uterus at the level of the isthmus and internal os. The artery then ascends alongside the uterine body and anastomoses with the ovarian artery. It also branches to the cervix and upper vagina,
which anastomoses with the ascending vaginal artery (Ellis 2011). The uterine vein accompanies the artery and drains into the internal iliac vein. It also communicates with the ovarian vein and with veins of the vagina and bladder via the pelvic venous plexus (Ellis 2011).

1.6 Endometriosis - A Disease of the Endometrium

It has been suggested that the immune disposal system removes ectopic endometrial cells and prevents their implantation and development into endometriotic lesions in healthy women. This process may be facilitated by endometrial cell apoptosis which ordinarily increases at the end of the menstrual cycle but is decreased significantly in women with endometriosis (Gebel M et al. 1998; Paul Dmowski & Braun 2004). In healthy women, disseminated endometrial cells in ectopic sites are programmed to die and are easily removed by the immune system. Similar to the eutopic endometrium of women with endometriosis, a deficient in-cell mediated immunity and/or a decrease in cell apoptosis may likely lead to the survival and implantation of cells into lesions (Paul Dmowski & Braun 2004). Many studies have reported that endometriotic ectopic lesions differ from those of the eutopic endometrium (Braun & Dmowski 1998; Gurates & Bulun 2003; Paul Dmowski & Braun 2004) in how they grow and survive. Moreover, it has been inferred that anomalies of eutopic endometrium probably precede and predispose the development of endometriotic lesions in ectopic sites (Al-Jefout, Tokushige, et al. 2009).
1.7 Theories of Endometriosis more focused on adolescents

The aetiology of endometriosis is complex and not completely understood, with many proposed theories explaining the different mechanisms involved. To date no single theory has been able to explain all cases of endometriosis, particularly in the adolescent population but all the proposed theories help to explain some aspect of the disease. Thus, the condition may arise from a combination of the major theories, consisting of the retrograde menstruation theory, the vascular/lymphatic dissemination theory, metaplasia of coelomic epithelium theory, the induction theory, and the embryonic rest theory (Seli et al. 2003). The types and frequencies of pathogenetic mechanisms involved in adolescent postpubertal/ premenarche endometriosis may have different aetiologies compared to adult endometriosis (Laufer 2012; Song & Advincula 2005).

1.7.1 Retrograde menstruation/ transplantation theory

The most commonly accepted theory is that of Sampson (1927), who proposed the implantation or retrograde menstruation theory. This theory suggests that during menstruation, viable endometrial cells left from the shedding of the endometrium, reflux through the fallopian tubes, thereby gaining access to, and implanting on the surrounding pelvic viscera (Sampson 1927). The disadvantage of this theory is that most normally menstruating women experience some retrograde menstruation with each cycle, thus, this theory lacks the explanation as to why certain women are predisposed while others are protected (Song & Advincula 2005). Furthermore, this theory is only supported assuming that endometriosis mostly occurs in the dependent portion of the pelvis. In the adolescent population, obstructive congenital anomalies of the female reproductive tract that
encourages retrograde flow has been associated with endometriosis (Sanfilippo 1997; Schifrin et al. 1973). One study identified six adolescents with müllerian (embryonic ducts which give rise to the genital passages in the female) anomalies (Schifrin et al. 1973), where the youngest patient was a 12 year old female with vaginal atresia and bicornuate uterus with developed hematocolpos; an accumulation of menstrual blood in the vagina. This was likely followed by retrograde flow leading to her endometriosis. There has been evidence that reparation of this type of obstructive abnormality has been correlated with the resolution of endometriosis (Sanfilippo 1997), however this is not true in all cases.

1.7.2 The induction/ coelomic metaplasia theory

The coelomic metaplasia theory was first proposed by Iwanoff and later propagated by Meyer (Metzger & Haney 1989). Unlike most of the other theories, the induction/coelomic metaplasia theory is among those proposing a non-uterine origin of the disease. This theory suggests that the coelomic epithelium is the common ancestor of endometrial and peritoneal cells, thus allowing transformation of normal peritoneal tissue to ectopic endometrial tissue (Burney & Giudice 2012). The ‘transformed’ peritoneal epithelium may be caused by chronic inflammation, chemical irritation from refluxed menstrual blood, or hormonal stimuli (Iwanoff 1898; Meyer 1924). This is a theory based upon embryologic studies indicating that all pelvic organs, including the endometrium, are derived from cells that line that coelomic cavity (Vinatier et al. 2001).

Further support for this theory developed from observations made from endometriosis in the adolescent population of premenarchal girls with some breast development (Laufer 2000; Batt & Mitwally 2003). The retrograde menstruation theory cannot explain the
presence of endometriosis seen in case studies involving adolescents, as these girls have not yet had menarche and thus cannot have had retrograde menses. In addition, further strengthening of this theory was seen in a recent case study involving a 20-year old girl with Mayer-Rokitansky-Küster-Hauser syndrome (a condition causing the vagina and uterus to be under developed or absent), who presented with increasing pelvic pain and underwent laparoscopy (Mok-Lin et al. 2010). Uterine, cervical, vaginal, and tubal agenesis were confirmed and upon laparoscopy endometriosis was further identified and destroyed. This case of endometriosis in an adolescent with complete uterine agenesis supports the induction/coelomic metaplasia theory (Mok-Lin et al. 2010).

1.8 Pathology and Appearance of Endometriosis

Endometriotic lesions in the pelvic cavity can be categorised into three types shown in Figure 1.3: endometriotic and peritoneal implants (Images A and B), deep infiltrating or adenomyomatous disease (Image C) and endometriomas (Image D) (ASRM 1997). Though these types of appearances are commonly seen, studies have also presented atypical lesions that do not fall into a specific category. It is also important to note that the occurrence of different types of lesions can often co-exist.
Figure 1.3: A: Shows an endometriotic implant (red lesion), adhesions, and hyperaemia in the peritoneum. B: Peritoneal implants, including red and blue-black lesions and adhesions. C: Extensive adhesions distorting the normal pelvic anatomy. D: An endometrioma adherent to the posterior uterus and distending the ovarian capsule.

Source: http://www.womenshealthsection.com

1.8.1 Superficial endometriosis

Most women have minimal or mild endometriosis, which is characterised by superficial implants and mild adhesions (ASRM 1997). The American Fertility Society classification system for endometriosis distinguishes superficial lesions as lesions that are no more than 5mm³ below the surface of peritoneum and the ovaries (Cornillie et al. 1990). The morphologic appearance of these endometriotic lesions can vary with several different presentations, generally appearing as superficial “powder burn” or “gunshot” lesions on the surface of the ovary, the serosal surfaces and/or the peritoneum (Images A and B, in Figure
1.3. The lesions can be black, dark-brown, or bluish puckered. They can also be seen as nodules or small cysts containing old haemorrhage surrounded by a variable extent of fibrosis. Atypical or subtle lesions are also common and include red implants, serous or clear vesicles (ASRM 1997). Other appearances include white plaques or scarring and yellowish brown peritoneal discoloration of the peritoneum. These peritoneal implants are further categorized into intraepithelial and sub-mesothelial lesions consisting of stromal and glandular tissue which are responsive to the hormones of the menstrual cycle. The adhesions that have been found in the ovary are haemorrhagic in nature and its severity worsens with the depth of the adhesion formation within the ovary (Giudice 2010).

1.8.2 Deep Infiltrating Endometriosis

Deep infiltrating endometriotic pelvic nodules (Figure 1.3, Image C) are mainly comprised of proliferative fibro muscular tissue with sparse glandular and stromal tissue with no surface epithelium (Giudice 2010). They are commonly seen in the uterosacral ligaments and rectovaginal septum. These nodules can sometimes extend greater than 5mm beneath the peritoneum involving the vaginal vault and sometimes the lesions of other abdominal organs such as the appendix, diaphragm, umbilicus, bladder, ureter and sciatic nerve roots (Vercellini et al. 2003; Fraser 2008). The depth of infiltration is related to the type and severity of symptoms. Unlike the peritoneal implants, this deep infiltrating type does not respond to the menstrual cycle hormones (Giudice 2010).
1.8.3 Ovarian Endometriomas

Apart from the typical surface lesions, there is another class of ovarian lesions referred to as the ovarian endometrioma or more commonly the ‘chocolate cyst’ because they are filled with old blood which is chocolate-like in colour (Figure 1.3, Image D). The colourisation is due to recurrent bleeding of the endometriotic implants into the cyst cavity. These lesions are organised and contain metabolised blood derivatives. They commonly appear as yellowish to marble-white shiny structures among younger women, whereas it is dark, fibrotic and vascularised in the older age group (Brosens et al. 1994; Brosens et al. 2004). With time, the endometriotic tissue slowly gets replaced by fibrotic tissue and the histological glandular appearance of the endometrioma disappears (Amer 2008). It is often a pseudocyst occurring due to the invagination of its cortical surface of the ovary and sealed into a cyst at the site of adherence (Brosens et al. 2004).

1.8.4 Lesions in Adolescents

Adolescent lesions are similar to adults in such a way that they vary in presentation. However they typically have clear, red, white, and yellow brown lesions more frequently than black or blue lesions (Appelbaum & Nentin 2010). Moreover, a study that compared endometriosis lesions in adolescents to those in adults found that red flame lesions were more common and powder burn lesions were less common (Davis et al. 1993). These results are consistent with the presumption that powder burn lesions represent older, more advanced implants (Demco, 1998). This also correlates with the results in Konnickx study where they found that pain symptoms correlated with the depth of the lesions (Porpora et al. 1999). Together, this suggests that the more superficial red and atypical lesions present in adolescents may not be as painful. However, it has become more clear that there are
other mechanisms responsible for the pain in endometriosis. There are cases of endometriomas in the adolescent populations that have been reported however, they are quite rare (Wright & Laufer 2010). Though it is important to note that most adolescent cases of endometriosis are diagnosed on the basis of history and physical examination and not laparoscopic visualisations of lesions, there may be many presentations of lesions left undetected.

1.8.5 Microanatomy of the lesion

A typical endometrial lesion has similar features to normal endometrium when viewed under the microscope. The histologic appearance consists of endometrial glands and surrounded by a small amount of dense “endometriotic” stroma with varying amounts of inflammation and fibrosis (Walter et al. 2001). Lesions can exhibit considerable variability in terms of glandular and epithelial cell morphology and vascular architecture (Machado et al. 2010). These variations can be attributed to the age and activity of the lesion (Berbic et al. 2010). For the purposes of understanding the lesions, many microanatomy studies have separated these lesions into three anatomical zones; the core of the lesions (glandular epithelium with the stroma), the α-smooth muscle actin (α-SMA) reactive interstitium and the exterior zone (Odagiri et al. 2009; Berbic et al. 2010).

1.9 Sites of Occurrence

Endometriosis is characterised by ectopically implanted endometrium which has similar histopathological and physiological response as that of the eutopic endometrium. The most common sites for ectopic endometriotic lesions are in the pelvis, particularly in the gravity
gradient dependent areas, followed by abdominal sites (Jenkins et al. 1986). In the pelvic cavity endometriotic lesions are most commonly found on the peritoneal surface of the ovary (most common), fallopian tubes, the uterus, the cervix, uterosacral ligaments such as the broad or round ligament, the pouch of Douglas, bladder peritoneum and rectovaginal septum (Fraser 2008) as shown in Figure 1.4. Abdominal sites such as the pleura, lungs, brain, and axilla have been documented, although these occurrences are rare (Giudice 2010; Fraser 2008).

**Figure 1.4:** Common sites of endometriosis

*Source: http://www.familyhealthonline.ca*

There are limited studies showing the frequent lesion sites for endometriosis in adolescents. However, a retrospective analysis of 63 cases of adolescent endometriosis in China found that of the 63 cases, 55 cases of lesions were found on the ovaries, 18 were found on the rectovaginal pouch, and 20 cases involved lesions of the uterosacral ligaments (Yang et al. 2012).
1.10 Clinical Concepts

The management of endometriosis encompasses two broad entities; investigations and treatment.

The approach to diagnosing and treating endometriosis is eliciting relevant clinical history followed by an appropriate clinical examination.

1.10.1 Symptoms, clinical presentation

Adults with endometriosis commonly have cyclic pain associated with chronic (lasting ≥ 6 months) pelvic pain, dysmenorrhoea, dyspareunia, a pelvic mass, infertility, deep pelvic pain and lower abdominal pain with or without back pain. The pain can be unpredictable and intermittent throughout the menstrual cycle or it can be continuous. It can also be dull, throbbing, or sharp, and even exacerbated by physical activity (American College of Obstetricians and Gynaecologists 2004). Pain often worsens with time and can change in character; women have reported burning or hypersensitivity, symptoms which are suggestive of endometriosis having a neuropathic component (Evans et al. 2007). Symptoms of endometriosis can overlap with those of several other gynaecologic conditions for example pelvic inflammatory disease, pelvic adhesions, ovarian cysts or masses, and adenomyosis (Giudice 2010). They can also coincide with non-gynaecologic conditions and factors such as irritable bowel syndrome, inflammatory bowel disease, interstitial cystitis, myofascial pain, depression, and a history of sexual abuse (Giudice, 2010), further challenging the diagnosis.

In contrast, adolescents with endometriosis frequently have both acyclic and cyclic pain (severe, progressive dysmenorrhoea). However, isolated cyclic pain is the least commonly presented (Laufer et al. 1997). Bowel symptoms inclusive of rectal pain, constipation,
painful defecation that may be cyclic, and rectal bleeding are also common as well as bladder symptoms such as dysuria, urgency and hematuria. Whereas, ovarian endometriomas and infertility are rare in adolescents (Laufer et al. 2003; Dessole et al. 2012).

1.10.2 Diagnosis

It has been speculated that with earlier diagnosis of endometriosis in adolescent, there may be a decrease in the length of time between patient presentation and clinical diagnosis, which currently averages 6.7 years (Nnoaham et al. 2011). In addition, earlier diagnosis and treatment of this disease will slow disease progression, possibly decreasing the adverse long-term effects of the disease such as chronic pain, endometriomas, and infertility, thus improving the quality of life of adolescents and women with this disorder (Nothnick 2001).

1.10.2.1 Method of diagnosis

The gold standard for diagnosis of endometriosis is surgical assessment by laparoscopy or laparotomy, followed by a histopathological examination of biopsied or excised lesions (Farquhar 2007). The severity is then scored using the revised classification of endometriosis system developed by the American Society of Reproductive Medicine (ASRM) (ASRM 1997). However, it is difficult to perform laparoscopic procedures on all patients with suspected endometriosis, particularly with adolescents. Figure 1.5 shows a flow chart of methods commonly used for diagnosis and treatment of endometriosis in adolescents. The diagnosis of patients by clinical patterns and detailed physical examination complemented with imagining methods is a fundamental step in the preliminary investigation of endometriosis, particularly in the adolescent. It provides the
ability to identify adolescents at high risk and those that require further evaluation, reducing the need of invasive investigations (Kafali et al., 2004).

Obtaining a patient’s full medical history helps to determine whether their symptoms are due to other causes. Following patient history, a relevant physical examination is essential to determine the aetiology of the pain and rule out an ovarian tumour or anomaly of the reproductive tract (Laufer et al. 2003). Although it is important, it may not be possible to perform a complete pelvic examination in all adolescents. For adolescents who are not sexually active, a rectal-abdominal examination may be better tolerated than vaginal-abdominal. Imaging methods, such as transvaginal ultrasonography and magnetic resonance imaging (MRI) should be performed to help a limited physical examination and identify/exclude causes of abdominopelvic pain other than endometriosis.
Figure 1.5: Diagnosis and treatment of adolescents with endometriosis. A flow chart of the method used.


Adapted from (Laufer et al. 2003; Armstrong 2011).
1.10.3 Treatment

Treatment of endometriosis in the past has been primarily focused on a straightforward ontological principle where surgical removal of endometriotic lesions was the preferred method for most surgeons specialising in the management of this disease (Gao et al. 2006). Although surgery has been seen to provide substantial relief, invasiveness, morbidity and complication risk aside, the recurrence of the disease remains a challenge where an estimated 21.5% of patients have relapse of the disease at 2 years (Guo 2009) and 40-50% at 5 years after the primary surgery (Guo 2009; Evers et al. n.d.; Martini et al. 2009).

Long term treatment of patients with chronic pelvic pain associated with endometriosis has also involved repeated courses of medical therapies (Idem 2008). This method of therapy is intended to reduce pain, through a variety of mechanisms, including inflammation, interrupting or suppressing cyclic ovarian hormone production, inhibiting the action and synthesis of oestradiol, and reducing or eliminating menses (Giudice 2010). In most medically treated patients, pain recurs within 6 to 12 months after completion of treatment (Kennedy et al., 2005).

Empirical medical therapy is commonly initiated for pain control without surgical confirmation of endometriosis. These therapies are intended to provide adequate analgesia and suppression of the activity of the lesion. Various medications including non-steroidal anti-inflammatory drugs (NSAIDS), oral contraceptive (OC) steroids, progestagens, agonists of gonadotropin releasing hormone (GnRH), as well as androgens (Lessey 2000; Valle & Sciarra 2003; American College of Obstetricians and Gynaecologists 2004) are used in clinical practice to address these symptoms. Intrauterine device (IUD) containing
progestin, such as Mirena, is an agent used to suppress lesion activity and may be effective in reducing endometriosis-associated pain by reducing menstrual flow (Yeung 2014). The most commonly used analgesics are NSAIDS followed by codeine compounds and tramadol in acute exacerbations. Careful usage of opioids in intractable pain is also seen in clinical practice (Fraser 2008).

In adolescents, medical treatment of dysmenorrhea is appropriate prior to consideration of surgical intervention for diagnosis of endometriosis (Figure 2.1). Particularly in adolescents with dysmenorrhea and/or have difficulty participating in normal activities, are missing school or avoiding extracurricular activities due to pelvic pain. Cyclic combination hormonal therapy (CHT) and NSAIDS are reasonable approaches when the pain evaluation suggests a non-acute gynaecological source. Hormonal therapy should be given with NSAIDS (American College of Obstetricians and Gynaecologists 2005).

If the pain is unresolved with NSAIDs and hormonal treatment, further evaluation is needed to determine whether endometriosis is the aetiology of the pain (Figure 1.5). The use of GnRH agonist allows patients with chronic pelvic pain and a high probability of endometriosis to avoid a diagnostic surgical procedure. Empiric GnRH agonists are not used for adolescents 18 years of age or younger due to concerns of potential adverse long term effects on bone formation and bone mineral density (American College of Obstetricians and Gynaecologists 2005).
Surgical approaches to relieve endometriosis related pain can be used as first-line therapy or initiated after failed medical therapies. A definitive diagnosis should be established before administering further treatment to adolescent with persistent pain after a period of three to six months with medicinal treatment (Armstrong 2011). As mentioned previously, laparoscopy is the gold standard for diagnosis of endometriosis. Surgical approaches include excision, fulguration, or laser ablation of endometriotic implants on the peritoneum, excision or drainage or ablation of endometriomas, resection of rectovaginal nodules, lysis of adhesions, and interruption of nerve pathways (Giudice, 2010). It is essential that the gynaecologist performing the laparoscopy on the adolescent not only has experience operating on patients in this age group but is also familiar with the appearance of endometriosis implants in adolescents. It is also important to achieve a good cosmetic result in adolescents minimising scarring as much as possible (Laufer 2008).

1.11 Staging of endometriosis

There have been various attempts to classify the different stages of endometriosis so that the outcome of treatments could be compared with a certain degree of accuracy. Challenges have arisen due to its complexity, highly variable presentation and gaps in knowledge. To date, in spite of these attempts, it has not been feasible to evolve a classification system that would address the entire therapeutic requirements. The main needs of a classification would involve creating a common language, to enable specific diagnosis, standardise comparisons and to facilitate research applications (Adamson & Pasta 2010). The most commonly used system today is known as the revised American Society for Reproductive Medicine (ASRM). The ASRM provides a method of recording information about the disease extent and morphology
along with images to describe its appearance. The aim of the ASRM is standardised recording of intra-operative anatomical pathological findings and thereby possibly predicting the fertility outcome after treatment (ASRM 1997). Thus from the revised ASRM staging of endometriosis is shown in Figure 1.6

<table>
<thead>
<tr>
<th>Stage</th>
<th>Disease</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Minimal</td>
<td>A few superficial implants</td>
</tr>
<tr>
<td>II</td>
<td>Mild</td>
<td>More and slightly deeper implants</td>
</tr>
<tr>
<td>III</td>
<td>Moderate</td>
<td>Many deep implants, small endometriomas on one or both ovaries, and some filmy adhesions</td>
</tr>
<tr>
<td>IV</td>
<td>Severe</td>
<td>Many deep implants, large endometriomas on one or both ovaries, and many dense adhesions, sometimes with the rectum adhering to the back of the uterus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peritoneum</th>
<th>Endometriosis</th>
<th>&lt;1 cm</th>
<th>1-3 cm</th>
<th>&gt;3 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Deep</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ovary</th>
<th>Adhesions</th>
<th>&lt;1/3 enclosure</th>
<th>1/3-2/3 enclosure</th>
<th>&gt;2/3 enclosure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rt filmy</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dense</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Lt filmy</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dense</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16. Staging: Stage I (minimal): 1-5; stage II (mild): 6-15; stage III (moderate): 16-40; stage IV (severe): > 40*

**Figure 1.6:** AFS classification of Endometriosis (Agarwal & Subramanian 2010)
A systematic inspection of the pelvis is encouraged in a clock-wise or counter clockwise manner. The appearance, number, size, type and location of endometriotic lesions are noted and scores are assigned for these lesions based on the predetermined weighted point system for these parameters. These scores are added up to arrive at the aggregate score, which will determine the stage.

1.11.1. Limitations of Revised ASRM staging

Although the revised ASRM staging is useful in determining the burden of the disease and management, this system has many serious limitations. The staging system does not correlate with the severity for pain or with a woman’s chance of conception following therapy (The Practice Committee of the American Society for Reproductive Medicine 2006). Thus, it ineffectively predicts the outcomes for treatment. The poor predictive ability is related to the arbitrary assignment of a point score for the observed pathology and the random cut off points that establish the stage of disease (The Practice Committee of the American Society for Reproductive Medicine 2006). Due to the variability in presentations, it is unlikely that any accurate staging system will be provided in the near future, at least not until a better understanding of the pathophysiology of endometriosis-associated infertility occurs.
CHAPTER 2 – NEUROGENESIS IN ENDOMETRIOSIS

Neurogenesis is the process by which neurons are generated from neural stem cells and progenitor cells (Eriksson et al. 1998). For a better understanding of neurogenesis, a basic explanation of the nervous system is needed.

2.1 The Nervous System

The nervous system is among the smallest of organ systems in terms of body weight; however, it is by far the one with the most complexity. Along with the endocrine system, the nervous system controls and adjusts the activities of other systems and maintains homeostasis (NCBI 2001). The nervous system can be divided into two anatomical subdivisions: the central nervous system (CNS) and the peripheral nervous system (PNS).

The CNS encompasses the brain and spinal cord, responsible for integrating, processing, and coordinating sensory input and motor output. It is also accountable for higher functions such as intelligence, memory, learning and emotion (Martini et al. 2009). The PNS includes all of the neural tissue outside the CNS. The PNS provides sensory information to the CNS and carries motor commands from the CNS to peripheral tissues and systems. The PNS is further divided into two subdivisions, the afferent division brings sensory information to the CNS and the efferent division carries motor commands to muscles and glands. The two subdivisions are further divided into somatic and visceral components which innervate the lower abdomen and pelvis with associated viscera, muscles, bone, and skin. The afferent division carries information from somatic sensory receptors which monitor the innervated areas. The efferent division includes the somatic nervous system which controls skeletal muscle contractions and the
autonomic nervous system which is further divided into the sympathetic and parasympathetic nervous systems. The sympathetic nervous system results in vasoconstriction resulting in increased blood pressure whilst the parasympathetic nervous system stimulates glandular activity and smooth muscle and relaxes sphincters (Sherwood 2010). All the varied functions of the nervous system are executed by individual neurons that need to be kept safe and fully functional.

2.1.1 Neurons

Neurons are the function unit of the nervous system (Britannica 2014). Neural tissue contains two cell types: nerve cells called neurons and supporting cells, or neuroglia. Neurons are responsible for the transfer and processing of information in the nervous system. A neuron has a cell body, or soma, containing the nucleus and mitochondria, ribosomes, and other organelles. The cell body has branching dendrites bearing many fine processes called dendritic spines that deliver the information in the CNS to the neuron. The cell body is attached to an elongated axon that ends at one or more synaptic terminals used to communicate with another cell. Neurons can be classified into three functional groups: sensory neurons, motor neurons, and interneurons (Martini et al. 2009).

1) Sensory neurons

Nearly all cell bodies of sensory neurons are located outside the CNS in the peripheral sensory ganglia. They form the afferent division of the PNS where their primary function is to deliver information to the CNS. Sensory neurons transmit information via different types of receptors broadly categorised into exteroceptors, proprioceptors and interoceptors (Martini et al. 2009).
2) Motor neurons

Motor neurons form the efferent division of the nervous system. They have short dendrites and long axons that relay messages from the CNS to the skeletal muscles, exerting a particular action (Martini et al., 2009).

3) Interneurons

Interneurons may be situated between sensory neurons and motor neurons. These neurons are located entirely within the brain and spinal cord. They are responsible for the analysis of sensory inputs and the coordination of motor outputs. The greater the response from a stimulus the greater the number of interneurons are involved (Gray et al. 1995).

2.1.2 Neuron Growth

Supporting cells, termed neuroglia isolate the neurons which provides the framework for support and helps maintain the intercellular environment needed to act as phagocytes. There are significant differences between the neuroglial cells of the CNS and that of the PNS. Neuron cell bodies of the PNS are typically clustered together in groups called ganglia that bundle together with other ganglia to form complex systems. The two major groups of ganglia are the dorsal root ganglia (DRG) and the autonomic ganglia. The DRG are located alongside the spinal cord, and contain the cell bodies of sensory (afferent) nerves, both somatic and visceral. Sensory ganglion cells in the dorsal root provide both central and peripheral directed processes and do not have synapses on their cell bodies. The peripheral processes of visceral afferent neurons are divided throughout the autonomic ganglia and plexuses.
Altogether the pelvic plexuses receive fibres from the sacral branches of the sympathetic trunk and parasympathetic input from the pelvic splanchnic nerves from the anterior primary rami of sacral splanchnic nerves S2-4 (Gibbins 2004). The pelvic plexuses extend ventrally toward the bladder and internal genital organs (Curtis et al. 1942; Wozniak & Skowronska 1967; Baljet & Drukker 1981). It should be noted that there is substantial evidence suggesting that the activity of some final motor neurons in the pelvic plexuses can be directly influenced by peripheral enteric neurons of the gastrointestinal tract (Luckensmeyer & Keast 1996). The details of the functional anatomy of the pelvic plexuses in humans are still incomplete and are best described in relation to the organs they innervate.

2.1.3 Uterine Innervation

The smooth muscle of the uterus is mostly innervated by the nerve cell bodies in the inferior parts of the pelvic plexuses, especially the paracervical ganglia. These ganglia lie in the broad ligament immediately dorsal to the origin of the uterine artery (Curtis et al., 1942, Davis, 1933). Some of the postganglionic neurons may have cell bodies in the lumbar paravertebral ganglia or the superior hypogastric plexus (Bell 1972; Marshall 1970).

2.1.4 Peritoneal Innervations

The parietal peritoneum lining the anterior abdominal wall, derives its nerve supply from the afferent nerves of the lower six thoracic and first lumbar nerves (T10-L2) in the anterior abdominal wall (Gray et al. 1995; Roberts 2005; Ahluwalia et al. 2004). The diaphragmatic part of the parietal peritoneum is supplied by the phrenic nerve (C 3,4,5) in
the central region whereas the peripheral region is supplied by the lower six thoracic nerves. The visceral peritoneum is supplied by autonomic afferent nerves from the same nerve innervating the organ that it covers. This layer is sensitive to tension, muscle spasms, ischemia, and some unknown factors (Gray et al. 1995).

2.2 Pathophysiology of Pain in Endometriosis

Pain has been defined as “unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” by the International Association for the Study of Pain (Howard 2009). However, chronic pelvic pain has considerable variety in definition. Some authors use a restrictive definition excluding severe dysmenorrhoea (Howard 2009; Kontoravdis et al. 1996) and others recommend a broader definition that incorporates dysmenorrhoea, deep dyspareunia, and all other painful symptoms located in the pelvis (Carter 1995; Rapkin 1986; Vercellini et al. 1990).

Although there has been a lot of ongoing research, the mechanisms by which pain affects women with endometriosis remains poorly understood. Studies that have implicated an explanation of these pain mechanisms have been mainly consisting three groups: nociceptive, inflammatory, and neuropathic.

2.2.1 Nociceptive

Nociceptors are relatively unspecialised nerve cell endings that initiate sensation of pain. They transduce a variety of stimuli into receptor potentials, which in turn triggers afferent action potentials (NCBI 2001). Nociception is triggered as a result of noxious stimuli and
undergoes three main steps before the pain is perceived; transduction, transmission, and modulation. Transduction is a process by which the noxious stimuli are converted into a neurochemical signal at the nociceptive receptor level, by the neurons. This process is then followed by the transmission of the signal to the CNS. After which, the signal is then processed in the CNS and, is either lowered or amplified by modulation and perceived in the cerebral cortex as pain (Howard 2009).

### 2.2.2 Inflammatory

Inflammatory pain is perceived as a result of response to inflammatory process (Howard 2009). The presence of prostaglandins is an essential part of the inflammatory environment and they contribute to pain directly and through the stimulation of expression of other pain mediators such as histamine, serotonin, NGF and prostanoids. The precursor of prostaglandins is arachidonic acid and cyclooxygenase (COX) enzyme is responsible for the conversion into prostaglandin. Higher levels of COX are a characteristic of the inflammatory environment. Interleukins such as IL-1, IL-6 AND IL-8 are pro-inflammatory cytokines that play a major role in the production of prostaglandins. TNF-present in the inflammatory environment is also responsible for promoting prostaglandin levels, by stimulating the production of the enzyme prostaglandin synthetase. These pain mediators in the inflammatory environment stimulate nerve endings in the site and generate pain (Howard 2009).

### 2.2.3 Neuropathic

Neuropathic pain is defined as “pain arising as direct consequence of a lesion or disease affecting the somato-sensory system” by the Neuropathic Pain Special Interest Group of
the International Association for the Study of Pain (Geber et al. 2009). It may result as a consequence of tissue damage, inflammation or injury to the nervous system. The pain is often chronic, characterised by allodynia (pain due to a stimulus which does not normally provoke pain), hyperalgesia (an increased sensitivity to pain) and spontaneous pain (Moalem & Tracey 2006).

The two major attributes of neuropathic process in chronic pain state, especially pertaining to endometriosis are central sensitization and peripheral sensitization. Central sensitization refers to the phenomenon by which the neurons of the CNS become highly responsive for pain transmission. Several neuro-chemical changes in the CNS have been attributed for this effect. Peripheral sensitization refers to the alteration of nociceptive function where the threshold for activation is lowered and the responsiveness to suprathreshold level is increased.

2.3 Pain Mechanisms in Eutopic endometrial and peritoneal lesions of endometriosis

There are several studies that speculate the pain perceived in endometriosis could be due to nerve fibre expression, angiogenesis, immunology, and endocrinology in the eutopic endometrium and peritoneal lesions.

2.3.1 Eutopic endometrial anomalies

The term ‘eutopic endometrium’ is commonly defined as endometrium in the usual site lining the uterine cavity in women with endometriosis (Al-Jefout, Tokushige, et al. 2009). There is increasing evidence which suggests that the eutopic endometrium of women with endometriosis display fundamental abnormal changes compared to that of women without
endometriosis (Bondza et al. 2009). It is both proliferative and secretory eutopic endometrium that exhibit changes in endometriosis with heterogeneous responses (Brosens et al. 2012). The existence of fundamental changes in eutopic endometrium of women with endometriosis is supported by the presence of nerve fibres, angiogenic factors, immunological changes, and hormonal dysfunctions. Together, these dysfunctions have been speculated to play a central role in the pathogenesis of endometriosis and its manifestation.

2.3.2 Peritoneal lesions in endometriosis

Ordinarily, the immune disposal system removes ectopic endometrial cells and prevents their implantation and development into endometriotic lesions in healthy women. However, this process is facilitated by endometrial cell apoptosis which ordinarily increases at the end of the menstrual cycle but is decreased significantly in women with endometriosis (Gebel M et al. 1998; Paul Dmowski & Braun 2004). Similar to the eutopic endometrium of women with endometriosis, a deficient in-cell mediated immunity and/or a decrease in cell apoptosis will in all probability lead to the survival and implantation of cells into ectopic lesions (Paul Dmowski & Braun 2004). Many studies have reported that endometriotic ectopic lesions differ from those of the eutopic endometrium (Braun & Dmowski 1998; Gurates & Bulun 2003) in how they grow and survive. Moreover, it has been inferred that anomalies of eutopic endometrium probably precede and predispose the development of endometriotic lesions in ectopic sites (Al-Jefout, Tokushige, et al. 2009).
2.4 Neurogenesis

As previously mentioned, neurogenesis is the process by which neurons are generated from neural stem cells and progenitor cells (Eriksson et al. 1998). The innervation of the human uterus has been researched for centuries, dating back to 1685 (Wilissius 1680). Studies have demonstrated that sympathetic, parasympathetic and sensory nerve fibres innervate peritoneal lesions of endometriosis (Mechsner et al. 2009; Tokushige et al. 2006b). Following these studies and although controversial, similar nerve fibres in the eutopic endometrium of women with endometriosis were also detected using PGP 9.5 and neurofilament (NF) markers (Tokushige et al. 2007). Endometrial biopsy performed with hysteroscopy with a vaginoscopic approach (Di Spiezio Sardo et al. 2010) combined with assessment of nerve fibres may be an ideal method utilised for adolescents with suspected endometriosis (Dessole et al. 2012). However, currently, there exists no study that discusses the innervation of peritoneal lesions or eutopic endometrium of the adolescent.

2.4.1 Eutopic Endometrial Neurogenic Anomalies in Endometriosis

In normal women, nerve fibres can be seen in the myometrium, the endometrial-myometrial interface and sometimes in basal layer of the endometrium (Fraser et al. 2006). However, they do not exist in the superficial two-thirds of the endometrium in the normal human uterus (Coupland 1969; Jones et al. 2003; Tokushige et al. 2006a). A study conducted by Tokushige et al (2006) reported the epic finding of multiple small unmyelinated sensory C nerve fibres in the functional layer of eutopic endometrium of women with laparoscopically confirmed endometriosis. None of these nerve fibres were expressed in the functional layer of women without endometriosis (Tokushige et al. 2006a). This study also concluded that women with endometriosis also had a highly
significantly increased nerve fibre density in the basal layer of endometrium and in the myometrium, when compared with women without endometriosis.

The nerve fibres in women with endometriosis were stained in the functional layer by pan neuronal marker, protein gene product 9.5 (PGP 9.5), but not with the myelination marker, NF, suggesting that the nerve fibres seen in the functional layer were unmyelinated. The use of other specific immuno-histochemical neural markers showed that these nerve fibres were also found to express cholinergic (Vasointestinal peptide; VIP), adrenergic (neuropeptide Y; NPY) and sensory (substance P; SP and calcitonin gene-related peptide; CGRP) markers (Tokushige et al. 2007).

Following the Tokushige et al. study, a pilot study by Al-Jefout et al. went on to evaluate endometrial biopsy and curettage in detecting small nerve fibres in eutopic endometrium for diagnosis of endometriosis. Their study showed that small nerve fibres were immunohistochemically detected by PGP 9.5 in all endometrial biopsies and curettings from all 20 women with endometriosis and no nerve fibres were detected in endometrium taken from women without endometriosis (Al-Jefout et al. 2007). They reported a 100% specificity, sensitivity, and positive and negative predictive value (Al-Jefout et al. 2007).

To further assess the efficacy of immunohistochemical nerve fibre detection by PGP 9.5 in endometrial biopsy as a method of diagnosing endometriosis, Al-Jefout et al organised another study using this method in a double blind comparison with diagnostic laparoscopy. In this study, endometrial biopsies taken from 99 consecutive women presenting with
pelvic pain and/or infertility undergoing diagnostic laparoscopy were compared with surgical diagnosis (Al-Jefout, Dezarnaulds, et al. 2009). In the 64 women with laparoscopic diagnosis of endometriosis, the mean nerve fibre density in the functional layer of the endometrial biopsy was 2.7 nerve fibres per mm$^2$ (± 3.5SD). There was only one woman with endometriosis who had no detectable nerve fibres. They also reported six cases where the endometrial biopsy was positive but there was no definitive evidence of endometriosis at laparoscopy. This study reported a specificity of 83% and sensitivity of 98% with a positive predictive value of 91% and a negative predictive value of 96% (Al-Jefout, Dezarnaulds, et al. 2009).

To further validate this method of diagnosis a second study in the same issue of Human Reproduction by Bokor et al. showed that the endometrial density of small nerves was approximately 14 times higher in 20 women with minimal to mild endometriosis vs 20 women with a normal pelvis. However, their study used a combined analysis of neural markers PGP 9.5, VIP and SP for nerve fibre detection with 100% specificity, 95% sensitivity and 97.5% accuracy (Bokor et al. 2009).

The conditions or molecular stimuli that may cause nerve fibres to grow into the eutopic endometrium in women with endometriosis remains unclear. In women with endometriosis, the neurotropin, nerve growth factor (NGF) and its two receptors tyrosine kinase -A (Trk-A) and p75, are present in eutopic endometrium and have been speculated to have an effect on nerve fibre growth (Kimpinski & Mearow 2001; Jones et al. 2003). In recent years, it has become apparent that NGF also plays an essential role in mediating
neuropathological and non-neuropathological pain (Apfel 2000). The neurotropic action of NGF is induced by binding to a high affinity receptor, Trk-A and a low affinity receptor, p75 (Braun et al. 2000). This induces receptor autophosphorylation and induction of intracellular signalling pathways, resulting in diverse biological effects (Kaplan et al. 1991).

NGF has been found to be strongly expressed in endometrial glands, stromal cells, blood vessels and nerve fibres in both the function and basal layers of the endometrium of women with endometriosis. It is also strongly expressed in smooth muscle cells, blood vessels and nerve fibres in the myometrium of women with endometriosis. Conversely, NGF is not seen in the functional layer of endometrium in women without endometriosis. Moreover, in normal myometrium it is moderately expressed in blood vessels and nerve fibres (Tokushige et al. 2007).

2.4.2 Neurogenesis in Endometriotic Peritoneal Lesions

The presence of nerve fibres in the peritoneal lesions of women with endometriosis has been implicated to contribute to the pain mechanisms of the disease. A study found that nerve fibre density was increased in the endometriotic lesions of women with endometriosis when compared to the normal peritoneum of women without the disease. They also found that the presence of unmyelinated nerve fibres were higher near the glands (Tokushige et al. 2006b).

To further strengthen nerve fibre expression in lesions, Mechsner et al. (2007) also documented that approximately 74% of the lesions studied had nerve fibres in direct
contact with them. They also demonstrated neurotropic properties of nerve fibres at the lesion site by showing neural outgrowth and regeneration at the lesion site and not at the peritoneum away from the lesion site and in control group without endometriosis. This neurotropic property may have an effect on the nervous system and prompts us to believe that there could be a neuropathic component in pain generation (Mechsner et al. 2007).

A study of Nerve growth factor (NGF), found NGF levels to be raised in the lesion, particularly in the glandular area. NGF promotes expression of nociceptors and neurogenesis of sensory neurons, which may induce pain by nociceptive mechanisms (Anaf et al. 2002; Tokushige et al. 2006a). Interestingly, another conducted by Tulandi et al. (2001) demonstrated that the distance between the nerve fibres and the lesions were decreased in women with pain compared to women without pain in endometriosis.

2.5 Angiogenesis

Angiogenesis is the process in which new blood vessels form from pre-existing vessels. It is a normal and vital physiological process that occurs primarily in embryo development, wound healing and in response to ovulation. This process involves the migration, growth, and differentiation of endothelial cells, which line the wall of blood vessels. The process of angiogenesis is controlled by growth and inhibitory factors in the body that signal and stimulate both the repair of damaged blood vessels as well as the formation of new ones.

Ordinarily the effects of these signals function in a precise balance so that vessels are formed when needed. When this balance is disturbed, the result is a disproportionate amount of angiogenesis. This abnormal blood vessel growth, either excessive or insufficient is now
recognized as a common denominator underlying many deadly and debilitating conditions including cancer, skin diseases, diabetic ulcers, cardiovascular diseases, endometriosis and many other diseases that rely on the mechanism of angiogenesis.

2.5.1 Angiogenesis of Eutopic Endometrium in Endometriosis

During normal reproduction, cyclic angiogenesis is orchestrated by the endocrine system, providing signals for corpus luteum function, follicular maturation, endometrial growth, and extensive vascular remodelling of the endometrium (Jaffe 2000; Fraser & Lunn 2000). These changes are regulated by ovarian steroids, many growth factors and inhibitors (Fraser & Lunn 2000). A key player is vascular endothelial growth factor (VEGF) (Ferrara & Davis-Smyth 1997) which is both synthesized and secreted by endometriotic cells (Shifren et al. 1996; Rocha et al. 2013).

In normal eutopic endometrium, significant angiogenesis takes place on a regular basis following the proliferative phase of menstruation (Gambino et al. 2002) where the derivation of new blood vessels from circulating endothelial progenitor cells (EPCs) occurs. This process is not only vital to the pathogenesis of endometriosis but it has been shown that abnormal levels of angiogenesis occur in the eutopic endometrium of women with endometriosis. VEGF is among the most potent and specific angiogenic factors. It causes endothelial cell proliferation, migration, organization into tubules, and enhanced permeability; all of which participate in the angiogenic process (Mueller et al. 2000) contributing to the growth of endometriosis.
Presently, there is limited data about angiogenesis specific to adolescent endometriosis. However, there are many studies with anti-angiogenic drugs that hold the promise of pelvic pain relief with the aim of successful achievement of pregnancy in infertile patient (Rocha et al. 2013). Since the majority of adolescents with endometriosis suffer from dysmenorrhea, it is logical that this method of treatment will target this population preventing onset of latter symptoms such as infertility.

2.5.2 Angiogenesis in Endometriotic Peritoneal Lesions

Neo-angiogenesis as the result of cytokine and VEGF secretion play an important role in the establishment of ectopic endometrial growth (Di Carlo et al. 2009). VEGF promotes vascularisation and formation of a new blood supply that is essential to the ectopic lesion. It has also been speculated that ectopic lesions may develop through VEGF stimulation of angiogenesis associated with the initial ovarian activity preceding the menarche (Brosens et al. 2013). Interleukin 8 (IL-8) is another potent angiogenic factor that is found to be up-regulated in the peritoneal cavity of women with endometriosis. It may function to assist endometrial-cell adhesion to peritoneal and other ectopic surfaces (Luk et al. 2005). Consequently, angiogenic factors are essential in promoting neo-vascularisation and neurogenesis, thus ensuring viability of the ectopic lesion. They also interfere with efficient immune-cell adhesion and most likely contribute to the defective immune response in women with endometriosis (Matarese et al. 2003).

2.6 Immunology

In the normal endometrium, leukocyte populations undergo significant changes during the menstrual cycle. The products released following the activation of these leukocytes provide
immune protection for the mucosal surface and play a critical role in menstruation, embryonic implantation and the maintenance of early pregnancy (Klentzeris et al. 1995). There have been numerous investigations that have implicated that disruptions in the immune system response are fundamental in the pathogenesis of endometriosis (Paul Dmowski & Braun 2004). It is clear that the immune system is involved in endometriosis, however it is unclear whether its involvement is a primary response leading to the initiation, promotion, and progression of the disease or a secondary response to ectopic endometrial growth in an attempt to restore homoeostasis (Matarese et al. 2003).

2.6.1 Immunology of Eutopic Endometrium in Endometriosis

There is very limited research that has focused on the immune response in the eutopic endometrium of women with endometriosis with conflicting evidence in the differences in leukocytes densities. It is important to note that most studies to date discuss the density of leukocytes in women with endometriosis however; the differences in their function have not been investigated. This is due to the fact that the endometrial immune cell populations are difficult to study due to their functional diversity and migratory capacity. Multiple markers would be needed to characterize the functional capacity of certain leukocyte populations (Al-Jefout, Tokushige, et al. 2009).

Early studies have reported that there are no significant differences in the immune cell populations in the eutopic endometrium of endometriosis (Coupland 1969). However more recent studies have reported a range of subtle but significant differences in specific leukocyte subsets during the menstrual cycle (Hey-Cunningham et al. 2011). Research has shown that CD4+ T helper cells, γδ T cells, monocytes, immature CD1a+ dendritic cells,
Foxp3+ Tregs during the secretory phase, macrophages have been shown to be significantly increased in the eutopic endometrium of women with endometriosis (Hey-Cunningham et al. 2011). It has been speculated that these up-regulated immune cell types may be exerting immunosuppressive effects on newly recruited endometrial leukocytes, affecting their ability to effectively target shed endometrial fragments (Hey-Cunningham et al. 2011).

2.6.2 Immunology of Endometriotic Peritoneal Lesions

Similar to the eutopic endometrium, immune cell aberrations have been detected in ectopic endometriotic lesions in women with endometriosis. They have decreased levels of cellular immunity, including natural killer (NK) cell functions and BAX-positive peritoneal macrophages, which may hold importance for the survival and proliferation of the aberrant, ectopic tissue (Koninckx et al. 1998). There are increased densities of a range of immune cell populations that have been described in endometriotic stroma compared to the surrounding sub-peritoneal tissue and normal peritoneum (Kempuraj et al. 2004; Schulke et al. 2009; Tran et al. 2009). The densities of immune cell populations at lesion site are much greater compared to matched eutopic endometrium (Chiang & Hill 1997; Jones et al. 1998; Schulke et al. 2009).

2.7 Endocrinology

The concept that endometriosis is an oestrogen dependent disorder has been well supported by molecular evidence (Kitawaki et al. 2002; Burney & Giudice 2012). However, other hormones such as progesterone, retinoic acid, and androgens have been found to affect the endometrial and endometriotic cell proliferation and counteract the oestrogen action (Jones et al. 1995). In fact,
more recently, the concentration of hormonal influence has shifted towards endometriosis being more of a progesterone resistance than an oestrogen dependent disease.

Endometriotic tissue proliferates in response to systemic oestrogens. It has been observed that the appearance of lesions is related to menstrual cycles, oestrogen action and the reduction of oestrogen effects. This concept has been supported by the fact that after menopause the disease seems to be reduced. Endometriotic cells have also been found to have their own ability to produce oestrogen, which helps enhance the growth of the diseased tissue (Jones et al. 1995). Studies have detected that there is increased activity of the aromatase enzyme and decreased expression of 17β Hydroxysteroid dehydrogenase type 2 (17β HSD2) (Jaffe 2000). Aromatase is the key enzyme in converting androstendione C19 into oestrogen. Aromatase expression and activity are absent in normal endometrium but not in the eutopic or ectopic endometrium of women with endometriosis (Bulun et al. 2001; Brosens et al. 2012). 17β HSD2 is an enzyme that is responsible for the inactivation of oestradiol to oestrone. Its function seems to be deficient in with endometriosis. The combination of these disruptions can be seen in Figure 2.1
Figure 2.1: Local oestrogen production in endometriotic lesions and eutopic endometrium

(Burney & Giudice 2012)

From Figure 2.1 we can see that with the addition of aromatase, the conversion of androstendione to oestrone is increased. This in combination with deficient $17\beta$ HSD2, the inactivation of oestradiol to oestrone is reduced. As the levels of oestradiol are higher, growth factors, prostaglandin (PGE$_2$) and NGF are also increased, leading to increased levels of adhesion, proliferation, anti-apoptosis, invasive phenotyping, neurogenesis, neuronal sprouting and inflammation. It is also important to note that PGE$_2$ also stimulates aromatase adding to this positive feedback mechanism. The combination of these factors help to sustain the life of the endometriotic tissue, ultimately leading to the pain seen in patients (Huhtinen et al. 2012).

It has been found that endometriotic lesions exhibit an overall reduction in progesterone receptors (Kim et al. 2001). There is a dysregulation of progesterone responsive genes in the
luteal phase. An incomplete transition of endometrium from the proliferative to secretory phase has significant molecular implications towards enhancing survival of refluxed endometrium (Burney & Giudice 2012). Therefore, furthering the perception that endometriosis is shifting from an oestrogen-dependent disease to a disorder is characterised primarily by progesterone resistance.
CHAPTER 3: HYPOTHESES AND AIMS

3.1 Hypotheses

In the subsequent chapters, the hypothesis that adolescents with endometriosis will also express nerve fibres in functional endometrium and peritoneal lesions will be investigated. Through a set of biopsied samples and immunohistochemical analysis, this was achieved by the following hypotheses which are applicable to adolescent patients with endometriosis:

1) Small unmyelinated nerve fibres known to contribute to pelvic pain in older women with endometriosis and their neuronal intensities will be detected in the eutopic endometrium, the peritoneal lesions or both by PGP 9.5, NGF, and Trk-A

2) The expression of nerve fibres and their intensity in the functional layer of the endometrium and peritoneal lesions will correlate with laparoscopically confirmed endometriosis.

3) The calculated nerve fibre density/ intensity will correlate with severity of disease.

4) There will be a reduced number of nerve fibres for patients on oral contraceptives/ combined hormonal therapy.

5) There will be no correlations between nerve fibre density/intensity and patient’s menstrual stage or age.

3.2 Aims

The aim of this study was to detect the expression of nerve fibres known to contribute to pelvic pain in older women with endometriosis in the eutopic endometrium and peritoneal lesions of adolescent females 19 years of age and below with laparoscopically detected and histologically
confirmed endometriosis. This study would contribute to the better understanding of pain mechanisms and to the pathophysiology of endometriosis in adolescents.

The specific aims are:

To characterise and study the presence of nerve fibres, detected with neuronal markers PGP 9.5, NGF and Trk-A in the functional layer of the endometrium and peritoneal lesions of adolescents with confirmed endometriosis.

1) To observe any relationships between PGP 9.5 nerve fibre density and NGF/Trk-A expression intensities
2) To investigate if nerve fibre densities/ neuronal intensities correlate with the severity of endometriosis
3) To observe any relationships between nerve fibre densities/ expression and menstrual stage
4) To observe any relationships between nerve fibre densities and medical treatment
5) To observe any relationships between nerve fibre densities/ expression and patient age
CHAPTER 4 – METHODOLOGY

4.1 Introduction

This research thesis is a part of a collaborative study between the Department of Obstetrics, Gynaecology and Neonatology, Queen Elizabeth II Research Institute for Mothers and Infants, The University of Sydney and The Women’s, The Royal Women’s Hospital in Melbourne.

The Royal Women’s Hospital in Melbourne was responsible for the identification of adolescent females (aged ≤ 20). They retrospectively identified uterine biopsies from adolescent patients whom had undergone laparoscopy for the investigation of pelvic pain between 2004 and 2009 from the archival histopathology storage facility as paraffin embedded blocks.

In the Department of Obstetrics Gynaecology and Neonatology, Queen Elizabeth II Research Institute for Mothers and Infants, The University of Sydney, the immunohistochemical analysis component was undertaken to detect the presence of and quantify key nerve fibres previously reported in adult women with endometriosis are also present in a adolescents with pelvic pain and determine how the expression relates to the diagnosis of endometriosis in young women. The biopsied adolescent tissues were to be immunohistochemically stained with antibodies previously shown to be markers of endometriosis in older women: Protein Gene Product (PGP 9.5), Nerve Growth Factor (NGF), and transforming tyrosine kinase (TrkA-8).
4.2 Ethics

The ethics component of this study was collaboratively submitted by Dr H. Kaur, Professor M. Hickey and Professor I. Fraser. This study has been approved by the Royal Women’s Hospital Research Committee and the Royal Women’s Hospital Human Research Ethics Committee; please refer to Appendix 1 for a copy of the approval letter.

4.3 Adolescent sample collection

At the women’s, the Royal Women’s Hospital in Melbourne, archival adolescent paraffin embedded uterine biopsies were selected from patients who had undergone laparoscopy for the investigation of pelvic pain between 2004 and 2009. These adolescent samples had laparoscopically visualised and histologically confirmed endometriosis. With these archived biopsies, records of the nature, severity and duration of the clinical symptoms had been recorded. The location and stage of endometriosis as well as any other clinical features were also recorded to the patient record.

From these collected Dilation and Curettage (D&C) samples, 74 women (aged ≤ 19 years) were identified. The adolescent samples were then de-identified with a study number for confidentiality of the participants and all clinical details were then entered into a database.

4.3.1 Selection criteria

**Inclusion criteria**

In order to collect the archived sample block for this study, the patient needed to meet the following criteria
1) ≤ 19 years old
2) Female
3) Is not pregnant at the time of procedure
4) Had undergone laparoscopy for the investigation of pelvic pain
5) Must have histopahtologically confirmed endometriosis

**Exclusion criteria**

1) Patients who could not participate in the study if they:
2) ≥ 19 years old
3) Had been histopathologically confirmed to not have endometriosis

**4.4 Histological preparation**

Embedded paraffin blocks were cooled for sectioning. The samples were cut into 4µm sections using a Leica Rm 2135 (Leica Microsystems Nussloch GmBH, Germany) microtome (Fig. 4.1). These sections were then transferred to water bath, preheated to 38-40°C and then transferred onto FLEX IHC microscope slides (Dako Denmark A/S, Glostrup, Denmark). They were then placed into the oven at 60°C for no more than 20-30 minutes to remove excess water and ensuring that the tissue fixed onto the slide. Six to eight sections were cut from each block and were mounted using this process. Afterwards, all slides were placed into freezer proof boxes and placed into the freezer until ready for immunohistochemical staining.
4.5 Histological staining and tissue identification

One slide from each sample was used for haematoxylin and eosin (H&E) staining for the identification of tissue type and latter assessment of tissue morphology by an experienced gynaecological histopathologist. H&E is the most widely used stain for medical diagnosis and is often the gold standard. Tissues were taken out of the freezer and brought to room temperature prior to H&E staining. They were deparaffinised and dehydrated through xylene, graded alcohols and placed into tap water (Table 4.1). They were then stained with H&E, rehydrated with graded alcohols and xylene and then mounted onto slides using FRONINE Ultramount no. 4.
Table 4.1 Method and times of deparaffinisation, dehydration and rehydration. The slides were first deparaffinised and dehydrated and then dehydrated again in the reverse steps and mounted after H&E staining.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time</th>
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<tr>
<td>Xylene</td>
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<tr>
<td>Xylene</td>
<td>5 minutes</td>
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<td>100% Alcohol</td>
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<td>95% Alcohol</td>
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<tr>
<td>70% Alcohol</td>
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After the tissues had been stained they were examined under the microscope to identify tissue types, with the help of Professor Peter Russell, specialising in gynaecological pathology at Douglas Hanly Moir Pathology (DHM). The tissues types were identified as well as any clinical morphology was noted, the samples were then separated into endometrial, peritoneal lesion and other tissue types. For the purpose of our study we only kept the patients who had either endometrial or both endometrial and peritoneal lesion tissue samples.

4.6 Immunohistochemistry (IHC)

Immunohistochemical staining methods involve the directed action of an antibody (Ab) against a particular binding site on the antigen (Ag), also known as an epitope. The antibody is formed in response to this epitope and its interaction is known as the antigen-antibody complex. This interaction between the antigen and its antibody is then made visible by molecules such as
enzymes, chromogens and fluorochromes which are then conjugated either directly or indirectly with the antibody. Depending on the number of specific binding sites and the method used (direct or indirect) the binding signal can thus be amplified and then visualized (Ramos-Vara 2005). Described in this section are the use of antibodies in IHC and their applications in the methodology of my study.

4.6.1 Use of Antibodies in IHC:

Antibodies are primarily glycoprotein structures found in the gamma globulin fraction of serum, they are thus known as immunoglobulins (Ig). There are five classes of these immunoglobulins, IgG, IgM, IgA, IgD and IgE. These immunoglobulins have different amino acid composition, structure, size, weight and function. In IHC staining, IgM and IgG are mainly used as they are produced as part of the primary and secondary response respectively (Ramos-Vara 2005).

The shape of an Antibody is typically Y-shaped with two identical heavy chains (H) and two identical light chains (L) that are held together by disulphide linkages (Figure 4.2). The H chain’s structure is different between the classes of antibodies that help to identify the class of immunoglobulins. The light chains contain the fragment antigen binding (Fab) portion of the molecule that bind to the antigen (Hayat 2002; Ramos-Vara 2005). While the heavy chains contain the fragment crystalline (Fc) portion of the molecule which is responsible for complement or chromogen binding. IgG has two gamma heavy chains, IgA consist of two alpha heavy chains. The two L chains however can either be one of two Kappa (K) or Lambda (L) and are independent of the class of Ig. Thus even if IgG has two K chains or two L chain, it will definitely have two gamma H chains (Ramos-Vara 2005).
Each heavy and light chain is further divided into regions which are constant (C) and Variable (V). The C region is towards the carboxyl (COO-) terminal of the amino acid.

Figure 4.2 Schematic of a typical antibody molecule
Source: www.eBioscience.com (2014)
chain whilst the V regions are towards the amino (NH2-) terminal (eBioscience 2014). The variable region of the H and L chains thus help to make the antigen-binding site.

4.6.1.1 Polyclonal antibody

For the purpose of using antibodies in IHC and laboratory use, the antigen must first be purified by methods such as precipitation, centrifugation and electrophoresis. This is then injected into an animal of another species such as rabbit, goat, pig, sheep, horse etc. and is thus identified as “foreign” by that animal and a humoral response is mounted against this antigen. Thus as a result antibodies are formed against this foreign antigen with IgM being the first humoral antibody detected at approximately one week from injection. IgG usually develops by the second week but are predominant and usually survive for around three weeks before it is catabolized. Unless repeated boosters of antigen are given, this serum Ab level will slowly decrease. Thus what are produced are Polyclonal antibodies that have slightly different specificities for the different epitopes on the antigen. With Polyclonal antibodies there thus is always a chance of cross reactivity. As the animal may have other Abs and proteins in its serum, this most often leads to non – specific staining in the tissue sections (Elias 2003; Ramos-Vara 2005).

4.6.1.2 Monoclonal antibody

Monoclonal antibodies on the other hand are immunochemically identical and thus will react with one specific epitope only. These are produced by cloning of plasma cells and fusing them to myeloma cells and culturing in-vitro and then screening for the antibody of interest (Fung et al. 1992). Monoclonal antibodies are thus far superior as:
• Non-specific antibodies are not present thus resulting in less background staining
• Variability is not present between batches
• Can provide an unlimited supply of Ab

However due to a cost effective and a high sensitivity approach monoclonal antibodies are not always chosen for IHC staining procedures. They are extremely costly and laborious to produce and result in possible reduced sensitivity of the epitopes. Monoclonal antibodies are not seen to work well in heavily fixed tissue on account of the above reasons (Ramos-Vara 2005).

4.6.2 Antibodies used in the study

For the purpose of this study, three antibodies were used (Table 4.2) to demonstrate the presence of nerve fibres and neuronal activity in the endometrial curettings of adolescent females with endometriosis. They were as follows:

4.6.2.1 Polyclonal rabbit anti-protein gene product 9.5 (PGP9.5) (Dako, Glostrup, Denmark): This antibody is a highly specific pan-neural marker which is used to detect all types of nerve fibres. It is highly expressed in vertebrate neurons and neuroendocrine cells. Although the limitation of this antibody is that it can weakly label epithilum, muscular tissue, connective tissue and serum.

4.6.2.2 Polyclonal rabbit anti-nerve growth factor (NGF) (Dako, Glostrup, Denmark): This antibody is used to show the expression of nerve growth factor in the tissue. Nerve growth factor (NGF) is a potent neurotrophic factor, which supports the growth and survivability of nerve and/or glial cells. This protein has nerve growth
stimulating activity and the complex is involved in the regulation of growth and the
differentiation of sympathetic and certain sensory neurons.

4.6.2.3 Tyrosine receptor kinase-A (Trk-A) (Dako, Glostrup, Denmark): A high
affinity nerve growth factor (NGF). The Trk proto-oncogene family contains four
members, Trk-A, Trk-B, Trk-C, and Trk-E, which are variably expressed throughout
the central and peripheral nervous systems. Trk-A is seen to bind to nerve growth
factor (NGF) and autophosphorylate. The presence of this kinase leads to cell
differentiation and may play a role in specifying sensory neuron subtypes and has
been linked to pain.

4.6.2.4 Optimisation of antibody dilutions

Previous studies of immunohistochemical nerve fibre detection used PGP 9.5
dilutions ranging from 1:1400 (Al-Jefout, Dezarnaulds, et al. 2009) to 1:900 (Bokor
et al. 2009). However, these dilutions did not show nerve fibres in our tissue samples
or any of the three control tissues known to have nerve fibres; colon, vaginal, and
uterine tissue. Since these studies, newer antibodies and machines became available.
For this reason, Dr Lawrence Young, Dako’s application specialist and I decided to
re-optimise the staining protocol. Using the manufacturer’s suggested dilution of
1:200 and a suggested dilution of 1:400 from previous scientists with similar studies
preceding my own, we performed an immunohistochemical analysis with control
tissues using these dilutions. A dilution of 1:400 gave us optimal results, whereas
1:200 had sub-optimal results with no clear contrast between tissue and nerve fibres.
To further validate this dilution, we proceeded with another immunohistochemical
run that included tissues from my study and these control tissues using a dilution range from 1:200 to 1:500. Again a dilution of 1:400 gave us the best results.

Similarly we used this method to optimise for NGF and Trk-A dilutions. However, we found that optimal NGF dilutions varied between functional endometrial tissue and peritoneal lesions. As a result we used an NGF dilution of 1:400 for endometrium and 1:200 for peritoneal lesions.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Identifies</th>
<th>Dilution</th>
<th>pH of Antigen Retrieval</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td>All types of nerve fibres</td>
<td>1:400</td>
<td>9</td>
<td>30 minutes</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor expression</td>
<td>1:400 (endometrium) 1:200 (peritoneal lesions)</td>
<td>9</td>
<td>45 minutes</td>
</tr>
<tr>
<td>Trk-A</td>
<td>A high affinity receptor for NGF</td>
<td>1:350</td>
<td>6</td>
<td>60 minutes</td>
</tr>
</tbody>
</table>

Table 4.2 Summary of antigen retrieval incubation, optimal dilution, pH and incubation time for each antibody used in this study. All these methods were performed at room temperature (20-23°C). Note: the optimal dilution for NGF was different for endometrial and peritoneal lesions tissue samples.

4.6.3 Deparaffinisation, rehydration and antigen retrieval

Slides were placed in a slide rack suited for the Dako PT Link (DakoCytomation, Carpinteria, CA, USA) (Figure 4.3), which allowed for the entire pre-treatment process of deparaffinisation, rehydrated and heat induced target retrieval to be combined into a 3-in-1 specimen preparation procedure. This also reduced time as the whole process was completed in one hour.
The PT Link can be used for both pH 6 and pH 9 antigen retrieval solutions depending on the antibody being used. Antigen retrieval is performed to reverse the changes caused by fixation. It prepares the tissue by breaking down the cross links created by fixation and exposing the epitopes on the target tissue for the antigen-antibody complex to form. The PT Link also helped to reduce human error that would have been lost in manual deparaffinisation and manual rehydration through graded alcohols.

![Figure 4.3: PT link (Manufactured at DAKO)](image)

The slide rack was inserted into pre-heated EnVision™ FLEX Target Retrieval Solution (Dako Denmark A/S, Glostrup, Denmark) (pH 6.0 or 9.0 depending on the tissue), the temperature was set to 95°C and the container was covered. The timer was set for 20 minutes; once complete the container with the retrieval slides were allowed to cool in the Dako PT Link for 30 minutes after which the solution was quickly decanted and the container was then placed in running tap water and slides were rinsed for 1 minute to remove all traces of the retrieval solution.
4.6.4 Autostainer

In this study we used The Dako Autostainer Plus (DakoCytomation, Carpentaria, CA, USA) which is an automated horizontal slide-processing system for staining formalin-fixed, paraffin-embedded tissues, frozen sections, cytospins, cell smears, and fine-needle aspirates. The unit consists of a slide processor, dedicated desktop computer, printer, and Seymour Labelling System (Figure 4.4). The Dako Autostainer Plus is a barcode-driven staining system with easy-to-use Windows-based software that allows for pre-programmed protocols and customized programs (Figure 4.7 and Figure 4.8).

![Autostainer Dako Autostainer Plus S3400](image)

**Figure 4.4.** Autostainer Dako Autostainer Plus S3400, (DakoCytomation, Carpinteria, CA, USA)  
(DAKO, 2014)

4.6.5 IHC Reagents

For the purpose of staining a number of reagents are used. Their roles and order in the staining procedure are described below:
4.6.5.1 EnVision FLEX Target Retrieval Solution, Mouse, High pH (pH 9,
Dakocytomation, Golstrup, Denmark): after deparaffinisation, antigen retrieval
was done to correct the changes caused by fixation. The slides were placed in plastic
racks and immersed in target retrieval solution and heated in a water bath at 99°C for
20 minutes. The target retrieval solution is diluted to the proportion of 1:10 with de-
ionised water for the procedure.

4.6.5.2 EnVision FLEX peroxidase-blocking reagent (Dakocytomation, Golstrup,
Denmark): Flex H₂O₂ (peroxidase) (Dakocytomation, Golstrup, Denmark). The
purpose of this step in the procedure is to avoid background staining caused by
endogenous peroxidase, pseudoperoxidase and alkaline phosphatase activity in the
tissue.

4.6.5.3 Protein Block (Dakocytomation, Golstrup, Denmark): This is an additional step
to eliminate background that may lead to erroneous interpretation or false-positive
results. It utilizes casein, a hydrophilic protein which has been shown to reduce
nonspecific binding of primary antibody and secondary reagents in
immunohistochemistry. This comes in a ready to use bottle.

4.6.5.4 Primary Antibodies (Dakocytomation, Golstrup, Denmark): The antibodies that
are being used: Protein Gene Product 9.5 (PGP 9.5), Nerve Growth Factor (NGF),
and Tyrosine kinase receptor – A (Trk-A). Each of the primary antibodies were
diluted according to their optimised dilution (Table 4.2).

4.6.5.5 EnVision Flex+/HRP (Dakocytomation, Golstrup, Denmark): This is used to
amplify the signal. It binds to the nonspecific region of the Ab forming a link
between Ab and the polymer. This comes in a ready to use bottle.
4.6.5.6 EnVision Flex DAB chromogen (Dakocytomation, Golstrup, Denmark): The chromogen binds to the labelled polymer at numerous sites amplifying the signal. The detection system consists of the Envision+ Flex polymer together with the chromogen 3, 3-diaminobenzidine tetrahydrochloride (DAB). It is prepared by adding 1µl of DAB chromogen for each ml of substrate buffer.

Each of the reagents are made based on the estimated calculation for each of the run cycles carried out by the Autostainer. This is based on the estimation that the reagent should cover the entire area of the tissue section. However, the drop zones of the Autostainer can be changed for each slide to reduce waste of reagents.

Figure 4.5 An exemple of the Reagent Map Layout used in the Dako Autostainer. The reagent map documents the rack positions of the reagents (FLEX H₂O₂, Protein block, EnVision Flex+/HRP, EnVision
Flex DAB chromogen and primary antibodies (PGP9.5, NGF, Trk-A). The buffer and the ionized water requirements for the staining run.

Using the optimised dilutions, the Autostainer was programmed to stain our slides using a customised protocol (Table 4.3).
Figure 4.6 An example of the Dako Autostainer Plus programming grid. The Autostainer can process a maximum of 48 microscope slides in a single staining run. Each slide can be stained using an independently designed protocol. After the slide information is entered and protocol template is selected, specific reagents and their dispense volume are assigned to each slide.
Table 4.3 Dako Autostainer Plus protocol, these procedural steps were all done at room temperature (20°C - 23°C).

<table>
<thead>
<tr>
<th>Step</th>
<th>Instruction</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slides were rinsed with buffer</td>
<td>5 mins</td>
</tr>
<tr>
<td>2</td>
<td>Slides were incubated by endogenous enzyme block: Flex peroxidase (100 µl)</td>
<td>5 mins</td>
</tr>
<tr>
<td>3</td>
<td>Slides were rinsed with buffer</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Slides were incubated by protein block (100 µl)</td>
<td>5 mins</td>
</tr>
<tr>
<td>5</td>
<td>Slides were blown by the blow mechanism to remove the protein block</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Slides were incubated with the primary antibody (100 µl)</td>
<td>30 mins</td>
</tr>
<tr>
<td>7</td>
<td>Slides were rinsed with buffer</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Slides were incubated by labelled polymer (100 µl): Envision+ Flex</td>
<td>30 mins</td>
</tr>
<tr>
<td>9</td>
<td>Slides were rinsed with buffer twice</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Slides were incubated by substrate (100 µl): Flex DAB chromogen</td>
<td>10 mins</td>
</tr>
<tr>
<td>11</td>
<td>Slides were rinsed with buffer</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Slides were removed from racks, placed into container and rinsed with water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in preparation for counterstain</td>
<td></td>
</tr>
</tbody>
</table>

4.6.6 Counterstaining

The principle of counterstaining is to stain the tissue surrounding the structure of interest with a contrasting colour, so that the structure is visible more clearly against a contrast. The sections were subjected to the protocol in Table 4.4:
Table 4.4. Counterstaining protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Filtered Mayer’s Haematoxylin (2 quick dips)</td>
</tr>
<tr>
<td>2</td>
<td>Immersed in a holding jar and run cold tap water on it until the purple colour disappears.</td>
</tr>
<tr>
<td>3</td>
<td>Turn on the hot water tap and run this hot water over the slides for around 1 min.</td>
</tr>
<tr>
<td>4</td>
<td>Check if the slides are blue. If not continue running hot water. This makes use of the fact that tap water is slightly basic and also explains why the red haematoxylin in step 1 turns purple when passed through running water.</td>
</tr>
<tr>
<td>5</td>
<td>Excess water is tapped off and the slides are sent through the graded alcohols (70%, 90% and 100% twice) so as to remove all water traces. Alternately it can be put in the oven for 30 minutes at 60°C after 100% alcohol to dry out any remaining water before immersing in xylene. This prevents its contamination with water droplets.</td>
</tr>
<tr>
<td>6</td>
<td>Mount in DPX ultramount and coverslip making sure to avoid any air bubbles.</td>
</tr>
</tbody>
</table>

4.6.7 Positive and Negative control

The presence of controls in each run is extremely important as the presence of a positive positive-control and a negative negative-control ensures accurate staining. Tissues such as appendix, endometrial-myometrial interface (uterus), vagina, breast carcinomas were used as positive controls and stained with the same protocol as the other slides (Figure 4.7). In addition, with each endometrial IHC run, an adolescent endometrial tissue (Figure 4.8)
histopathologically confirmed to have nerve fibres was used as a positive control. This approach was also used to the peritoneal lesion IHC runs, where we also included an adolescent peritoneal lesion tissue with nerve fibres (Figure 4.9) as the positive control. However, for the negative control instead of using 100µl of primary antibody to stain the slides, 100µl of negative control solution was added.

**Figure 4.7:** PGP 9.5 positive control – vaginal tissue (400x), close to epithelial layer. The black arrows show examples of nerve fibres, which have positively stained a dark brownish red.
Figure 4.8: PGP 9.5 positive control – adolescent endometrial tissue (400x), histopathologically confirmed to have positive staining of nerve fibres. The black arrows show examples of nerve fibres, which have positively stained a dark brownish red.

Figure 4.9: PGP 9.5 positive control – adolescent peritoneal lesion tissue (200x), tissue has been histopathologically confirmed to have positive staining of nerve fibres. The lesion is in the top left hand corner of this slide. The black arrow shows an example of positive nerve fibre staining, which has stained a dark brownish red.
4.7 Analysis

The analysis of the samples stained with PGP 9.5, NGF and Trk-A was done by standard microscopy.

4.7.1 Microscopy

The images of endometrial and peritoneal tissue sections were captured using an Olympus microscope BX51 and digital camera DP 71 (Figure 4.10). The images were captured in the fields of view required for the study; this was different for endometrial and peritoneal lesion samples.

For endometrial sections, fields of view were taken across the entire section whereas for the peritoneal tissue, the fields of view used for this study were images taken surrounding the lesions, specifically the stromal and peri-stromal zones around the lesions (Figure 4.11).

Figure 4.10: BX 51 Olympus microscopy (left) and Olympus DP 71 camera (right)
Source: http://www.olympusmicro.com
Figure 4.11: Example of different zones of the peritoneal lesions used during microscopy analysis with a representative of the 50x50µm grid. S= stromal zone and PS= peristromal zone.

Once the images needed were captured in the fields of view required for study an assessment of nerve fibre density was performed using Image Pro plus Discovery 4.5 (Media Cybernetics, MD, USA). The following procedure was carried out to assess the density of nerve fibres in sections stained by PGP 9.5, NGF and Trk-A.

4.7.2. Calculations of nerve fibre density

Once the images features were controlled at the original magnification (200x), an orthogonal grid mask was sketched above the original images. The sections of the grid
used were tiles of equal squares of 50µm per side (Figure 4.11). The grid was set at 50µm side using the scale drawn on the image using the camera attached to the microscope, as a legend.

For the analysis of the endometrial tissues, once the grid was in position, the number of squares covering the section was counted and the numbers of positive encounters were counted using manual tags. When in doubt the magnification was raised to 400 times to assess actual positive encounters with artefacts and other tissue components. The number of positive encounters per square millimetre (mm²) was calculated as follows:

**Endometrial samples**

Positive counts/mm² = \[
\frac{\text{Total no. of positive encounters across the section}}{\text{No. of squares in the section} \times 0.0025}
\]

Note: 0.0025 = the area of each square = 50 x 50µm² = 2500 µm² = 0.0025mm²

As for the peritoneal tissue samples, the analysis was performed in two distinct zones namely stroma and the region surrounding the stroma, described as peri-stromal region. The same method was applied however it was separated into the two zones instead the entire section.

Peritoneal tissue samples:

**Stromal zone**

Positive counts/mm² = \[
\frac{\text{Total no. of positive encounters across the section}}{\text{No. of squares covered by stroma} \times 0.0025}
\]
**Peri-stromal zone**

Positive counts/mm² = Total no. of positive encounters across the section

No. of squares covered by peri-stromal x 0.0025

Note: peri-stromal zone is the area immediately surrounding the stroma area.

The results were expressed as the mean (± SD) number of nerve fibres per mm². An independent observer, without any knowledge of the clinical parameters or other prognostic factors and me, carried out the counting procedure twice.

### 4.8 Statistical Analysis of Data

SPSS version 17.0 (SPSS Inc., Chicago, Ill) was used for the statistical analysis of the data gathered from microscopy and ACIS procedures.

Kolmogorov-Smirnov test was used to determine the distribution of the data. Depending on the distribution we used the following methods for analysis.

<table>
<thead>
<tr>
<th>Tests used for statistical analysis</th>
<th>Normal distribution</th>
<th>Skewed distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired or related samples</td>
<td>Paired samples t-test</td>
<td>Wilcoxon signed rank test</td>
</tr>
<tr>
<td>Independent samples</td>
<td>Independent samples t-test</td>
<td>Wilcoxon rank sum test</td>
</tr>
<tr>
<td>Correlation</td>
<td>Pearson’s correlation coefficient</td>
<td>Spearman’s correlation coefficient</td>
</tr>
</tbody>
</table>
CHAPTER 5: RESULTS

5.1 Specimen distribution

Our study began with 59 adolescent patients who had undergone laparoscopy for the investigation of pelvic pain at Women’s Hospital in Melbourne from 2004 to 2009. The age group ranged from 15 to 19 years old (mean: 18, median: 18). 37 patients were on combined oral contraceptive pill (COCP), 2 were on progestogens (Primolut, Depo-Provera), 3 were on non-steroidal anti-inflammatory drugs (NSAIDs), and 17 were not on COCP/progestogens/NSAIDs. All patients had been histologically confirmed to have endometriosis.

Table 5.1 Specimen distribution

<table>
<thead>
<tr>
<th>Specimen category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients that meet study criteria</td>
<td>59</td>
</tr>
<tr>
<td>Total number of relevant biopsied samples (N)</td>
<td>84</td>
</tr>
<tr>
<td>Total number of endometrial samples</td>
<td>54</td>
</tr>
<tr>
<td>Total number of peritoneal lesions</td>
<td>30</td>
</tr>
<tr>
<td>Total number of patients with endometrial samples</td>
<td>51</td>
</tr>
<tr>
<td>Total number of patients with peritoneal lesions</td>
<td>15</td>
</tr>
<tr>
<td>Total number of patients with both endometrial samples and peritoneal lesions</td>
<td>13</td>
</tr>
</tbody>
</table>

Of these 59 patients, 143 biopsied tissue samples had been retrieved from archives, however only 84 were histological suitable for this study, reducing our patient number to 53. Of these 84 tissues, there were 54 endometrial samples and 30 peritoneal lesions. There were 51 patients
with endometrial tissue, 15 with peritoneal lesions and 13 with paired samples. The study’s demographics are summarised in Table 5.1. For the purpose of this study, we decided to analyse by tissue samples rather than patient, making our sample size (N): 84.

### 5.2 Immunohistochemical results

The aim of the study was to investigate the expression of nerve fibres in the functional endometrial layer and peritoneal lesions of adolescents using three antibodies: protein gene product 9.5 (PGP 9.5), nerve growth factor (NGF), and protein tyrosine kinase A (Trk-A).

**Table 5.2** Overview of immunohistochemical staining results for antibodies, showing the percentage of tissue samples that were positive for nerve fibre expression and intensity (N=84)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Endometrium</th>
<th>Peritoneal lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glands</td>
<td>Stroma</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>33.3% (18 of 54)</td>
<td>70.0% (21 of 30)</td>
</tr>
<tr>
<td>NGF</td>
<td>68.5% (37 of 54)</td>
<td>14.8% (8 of 54)</td>
</tr>
<tr>
<td>Trk-A</td>
<td>42.6% (23 of 54)</td>
<td>22.2% (12 of 54)</td>
</tr>
</tbody>
</table>

Note: There is not a separate stroma and gland result for PGP 9.5 as the nerve fibre expression was found to be homogeneously spread throughout the entire tissue samples.

From Table 5.2, 33.3% (18 of 54) endometrial tissue samples and 70.0% (21 of 30) peritoneal lesion samples positively stained for nerve fibre expression detected by PGP 9.5. For NGF and
Trk-A there were two separate scores for staining, one in the glands and the other in the stroma. For NGF, 68.52% (37 of 54) endometrial tissues stained positively in the glands while 14.82% (8 of 54) had staining in the stroma. For the peritoneal lesion tissues, 50.0% (15 of 30) had staining in the glands while 17.0% (5 of 30) had staining in the stroma. For the last antibody, Trk-A, 42.6% (23 of 54) endometrial tissues had positively stained glands and 22.2% (12 of 54) tissues with staining in the stroma. For the peritoneal lesion tissue, 33.3% (10 in 30) had staining in the glands while 13.3% (4 in 30) had staining in the stroma by Trk-A.

5.3 Endometrial results (n=54)

5.3.1 Relationship between stromal and peristromal nerve fibre densities

A comparative analysis of the nerve fibre densities of the two regions in the endometrial tissue samples was performed. Figure 5.1 is representative of adolescent endometrial tissue with positive staining for PGP 9.5. The nerve fibre expression is scattered throughout the endometrial tissue, suggesting that there is no pattern of distribution between stromal and peristromal regions.
Figure 5.1 Microscopy image of sectioned adolescent endometrial tissue positively stained for nerve fibres with PGP 9.5 at a magnification of 40x. The darkened brown staining are nerve fibres.

Table: 5.3 Density of nerve fibres in endometrium with statistics (N=18, representative of endometrial tissue with PGP 9.5 stained nerve fibre)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Endometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean n.f. density mm$^2$</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>57.70</td>
</tr>
</tbody>
</table>

Note: the values were expressed as mean of nerve fibre (n.f) density with standard deviation (SD), interquartile range (IQR) and the range of the densities for adolescent patients who had positively stained endometrial tissue for PGP 9.5. A one-sample Kolmogorov-Smirnov test was performed and the test demonstrated that the values were normally distributed.
5.3.2 Relationship between stromal and glandular NGF levels and Trk-A

The relationship of NGF and Trk-A levels in the stroma and the glands of the endometrial tissues were explored. The intensity score was based on an ordinal scale of - , +, ++, +++.

The images below are representative of adolescent endometrial tissue with positive staining for NGF (Figure 5.2) and Trk-A (Figure 5.3).

![Microscopy image of sectioned adolescent endometrial tissue positively stained (brown areas) NGF at a magnification of 40x. The staining in the glands would be an example of intensity level of “++” and the stroma would be “-“ (no visible staining).]

Figure 5.2
Figure 5.3 Microscopy image of sectioned adolescent endometrial tissue positively stained (brown areas) Trk-A at a magnification of 40x. The staining in the glands would be an example of intensity level of “+++” and the stroma would be “++”.

Table 5.4: Correlations in NGF and Trk-A levels in the glands and stroma of endometrial tissue

<table>
<thead>
<tr>
<th>Antibody &amp; Score comparison</th>
<th>Spearman’s rho correlations for endometrial tissue</th>
<th>rs</th>
<th>p value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>Stroma score vs. gland score</td>
<td>0.346*</td>
<td>0.10</td>
<td>54</td>
</tr>
<tr>
<td>Trk-A</td>
<td>Stroma score vs. gland score</td>
<td>0.549**</td>
<td>0.00</td>
<td>53</td>
</tr>
</tbody>
</table>

Note: * Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed)
The relationships of NGF and Trk-A levels in the glands and stroma were explored for any correlations using Spearman’s rho test. For the NGF scores there is a significant positive correlation between the gland and stroma scores at the 0.05 level. NGF levels in the glands were significantly higher than stromal NGF levels (p value = 0.10).

This was similarly seen in Trk-A scores, which also had a significant positive correlation between glandular and stromal scores but at the 0.01 level. Trk-A levels in the glands were significantly higher than stromal Trk-A levels (p value <0.001).

5.4 Peritoneal lesion results (n=54)

5.4.1 Relationship between stromal and peristromal nerve fibre densities

Comparative analyses of the nerve fibre densities of the two regions in the peritoneal lesion tissue samples were performed. Figure 5.4 is representative of adolescent peritoneal lesion tissue with positive staining for PGP 9.5.
Figure 5.4 Microscopy image of sectioned adolescent peritoneal lesion tissue positively stained for nerve fibres (the darkened brown areas) with PGP 9.5.

Table: 5.5 Density of nerve fibres in stromal and peristromal regions with statistics

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Stroma</th>
<th>Peristroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median n.f. density mm$^2$ (IQR)</td>
<td>Mean n.f. density mm$^2$ (SD)</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>4.00 (18.50)</td>
<td>90.11 (130.81)</td>
</tr>
</tbody>
</table>

Kolmogorov-Smirnov Test | 0.020 | 0.054 |

Note: the stroma values were expressed as median of nerve fibre (n.f) density with interquartile range (IQR) and the peristroma were expressed as mean n.f. density with standard deviation (SD) due to the variation in distribution.
The nerve fibre densities of sections stained with PGP 9.5 were significantly skewed for the stromal region whereas the peristromal region was normally distributed as tested by the One-Sample Kolmogorov-Smirnov Test. There is also a trend towards a significant positive correlation between stromal and peristromal densities ($r_s = 0.42$, $p = 0.055$), suggesting that the nerve fibre density in the stroma increases in tandem with those in peristromal region.

### 5.4.2 Relationship between stromal and glandular NGF levels and Trk-A

Similar to the analysis of the endometrial tissue samples, the relationship of NGF and Trk-A levels in the stroma and the glands of the peritoneal tissues were explored using an ordinal scale of - , + , ++ , +++ . The images below are representative of adolescent peritoneal lesion tissue with positive staining for NGF (Figure 5.5) and Trk-A (Figure 5.6).

![Microscopy image of sectioned adolescent peritoneal lesion tissue positively stained (brown areas) NGF at a magnification of 40x. The staining in the glands around the lesion would be an example of intensity level of 2 and the stroma would be 0-1 (lightly stained).](image.png)
Figure 5.6 Microscopy image of sectioned adolescent peritoneal lesion tissue positively stained (brown areas) for Trk-A at a magnification of 40x. The staining in the glands around the lesion would be an example of intensity level of 3 and the stroma would be 2.

<table>
<thead>
<tr>
<th>Antibody &amp; Score comparison</th>
<th>Spearman’s rho correlations for endometrial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$</td>
</tr>
<tr>
<td>NGF Stroma score vs. gland score</td>
<td>0.401*</td>
</tr>
<tr>
<td>Trk-A Stroma score vs. gland score</td>
<td>0.705**</td>
</tr>
</tbody>
</table>
The statistical analysis showed that there was a significant positive correlation between the NGF glandular and stromal scores ($r_s = 0.35$, $p = 0.028$). It also showed that there was a significant positive correlation between the Trk-A glandular and stromal scores ($r_s = 0.71$, $p < 0.001$).

5.5 Comparative immunohistochemical analysis

Comparative analysis of all adolescent tissues samples $N=84$, were done with immunohistochemical markers along against clinical characteristics of patients such as medical treatment, severity of disease, menstrual stage and age.

5.5.1 Relationship of nerve fibre density and expression and medication treatment

Analysis between all adolescent tissues samples $N = 84$, inclusive of endometrial and peritoneal lesions was done to see if a correlation exists between nerve fibre expression and patient medical therapy. As this was a retrospective study, details of medical therapy had not been recorded therefore we had no indications of their length of medical treatment and when/if it was ceased.
Table 5.7: Correlations between nerve fibre expression and medical treatment in all tissue samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Medical treatment</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>COCP</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>n</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>40.9%</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>59.1%</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>53</td>
</tr>
</tbody>
</table>

Note: * COCP = combined oral contraceptive, Depo = Depo-Provera, NSAIDs = non-steroidal anti-inflammatory drugs, PGP 9.5 = Protein gene product 9.5

From the analysis (Table 5.7), of the 38 positively stained for nerve fibre expression with PGP 9.5, 25 samples were on COCP, 1 on Depo, 2 on NSAIDS, 1 on Primolut and 9 were not on any medical treatment. Figure 5.7 shows a comparative example of the effect of medications on nerve fibre expression using antibody PGP 9.5. 40.9% (n = 9) of specimens that were not taking any medications showed nerve fibre expression while 59.1% (n = 13) of specimens not taking any medications did not show nerve fibre expression. Those on COCPs, 47.2% (n = 25) showed nerve fibre expression, while 52.8% (n = 28) did not. For samples on Depo 33.3% (n = 1) showed nerve fibre expression while 66.7% (n = 2) showed none. For those on NSAIDs, 40% (n = 2) had positive nerve fibre expression while 60% (n = 3) showed none. Lastly, those that were taking Primolut, 100% (n = 1) showed nerve fibre expression. Figure 5.7 demonstrates that for each medical treatment category the number of patients with positive nerve fibre expression were
very close to the number of patients without nerve fibre expression. This suggests that medical treatment may not have had an effect on the detection of nerve fibres in this population.

Figure 5.7 Relationship between the effect of medical treatment and nerve fibre expression in adolescent tissue samples using PGP 9.5

5.5.2 Relationship of nerve fibre density and expression and severity of disease

The relationship between nerve expression and intensity was explored against the severity of the disease based on the clinical staging of endometriosis. As the stage of endometriosis is an ordinal value, rather than a continuous one, Spearman’s correlations were used for the analysis.
Table 5.8 Relationship of nerve fibres and stage of endometriosis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Severity of disease, endometrial staging</th>
<th>Spearman's rank correlation coefficient $r$, value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Stroma density</td>
<td>-0.29</td>
<td>0.070</td>
</tr>
<tr>
<td></td>
<td>Peristroma density</td>
<td>-0.060</td>
<td>0.797</td>
</tr>
<tr>
<td>NGF</td>
<td>Gland intensity score</td>
<td>-0.060</td>
<td>0.714</td>
</tr>
<tr>
<td></td>
<td>Stroma intensity score</td>
<td>0.023</td>
<td>0.888</td>
</tr>
<tr>
<td>Trk-A</td>
<td>Gland intensity score</td>
<td>0.010</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>Stroma intensity score</td>
<td>-0.049</td>
<td>0.632</td>
</tr>
</tbody>
</table>

For PGP 9.5, no statistically significant correlations were found between nerve fibre density expression in the stroma and peristroma region of the tissue samples and the severity of the disease (no p values < 0.05). Similarly for antibodies NGF and Trk-A, there were no correlations in the staining seen in the glands and stroma of the tissues and the disease severity (no p values < 0.05).
5.5.3 Relationship between nerve fibres and menstrual stage

5.5.3.1 Relationship of nerve fibre densities and expression and menstrual stage

The relationship between nerve fibre density in the stroma and the peristroma regions and menstrual cycle were explored.

Table 5.9 Nerve fibre density vs. menstrual stage

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Menstrual stage</th>
<th>Statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stroma density</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proliferative</td>
<td>35.54 (45.19)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>61.81 (68.30)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Peristroma density</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proliferative</td>
<td>99.56 (153.44)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>64.65 (43.20)</td>
<td>3</td>
</tr>
</tbody>
</table>

The density of nerve fibres did not differ significantly between samples from the proliferative stage or from the secretory stage (no p values < 0.05); therefore there were no correlations between the nerve fibre densities seen in the adolescent tissue samples and the menstrual stage they were in.

5.5.3.2 Relationship of nerve fibre intensities (NGF, Trk-A) and menstrual stage

The relationship between nerve fibre expression in the stroma and glands against menstrual stage were analysed using chi-square tests to look for any associations between the groups. We examined whether menstrual stage has a linear association with NGF expression.
Table 5.10 Nerve fibre expression vs. menstrual stage, statistics

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Statistics</th>
<th>Linear by linear association value</th>
<th>Degrees of freedom (df)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>Gland intensity score</td>
<td>1.176</td>
<td>1</td>
<td>0.278</td>
</tr>
<tr>
<td></td>
<td>Stroma intensity score</td>
<td>0.038</td>
<td>1</td>
<td>0.845</td>
</tr>
<tr>
<td>Trk-A</td>
<td>Gland intensity score</td>
<td>1.469</td>
<td>1</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>Stroma intensity score</td>
<td>0.699</td>
<td>1</td>
<td>0.403</td>
</tr>
</tbody>
</table>

There was no statistically significant association between patients menstrual stage and NGF expression (p value >0.05). Similarly, there was also no statistically significant association between patient’s menstrual stage and Trk-A expression (p value > 0.05).

5.5.4 Relationship of nerve fibres and patient’s age

For this comparative analysis, nerve fibre density was only taken into account as we wanted to see if there were any correlations in the presence of nerve fibres and patients age.

Table 5.11 Relationship of nerve patient’s age vs. nerve fibre density

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Spearman’s rho</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman's rank correlation coefficient $r_s$ value</td>
<td>N</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Stroma density</td>
<td>0.229</td>
</tr>
<tr>
<td></td>
<td>Peristroma density</td>
<td>-0.055</td>
</tr>
</tbody>
</table>

Note: For patient’s age, the calculated median: 18, mode: 18, and range: 4
Statistically analysis showed that the median age in this population was 18, with a mode of 18 in a range of 4. Using Spearman’s correlation, it was found that the age of the patients were significantly skewed and neither stroma density nor peristroma density had any correlations with age (p value >0.05).

5.5.5 Relationship of nerve fibre densities expression between the endometrium and peritoneal lesions

A comparative analysis was done to see if there was a correlation between patients who expressed nerve fibres in endometrial tissues also expressed nerve fibres in the paired peritoneal lesion tissue sample. A comparative analysis was done to see if there was a correlation between patients who expressed nerve fibres in endometrial tissues also expressed nerve fibres in the paired peritoneal lesion tissue sample. Initially we had 14 paired samples, however for the purpose of this study only the five tissue samples with positive nerve fibre expression in the endometrium were explored. Of these five samples, only three pairs had consistent positive nerve fibre expression in both their endometrial and peritoneal lesion samples. Using Fisher’s Exact test, the p value = 1.00, suggesting there was not a statistically significant association between having positive or negative nerve fibre expression in the endometrium and peritoneal lesion.

5.5.6 Relationship between nerve fibre densities and nerve fibre expression

The relationship between nerve fibre densities (PGP 9.5) and nerve fibre expression (NGF, Trk-A) was analysed for correlations for adolescent tissue samples that had positively stained for nerve fibres n=38.
Table 5.12 Statistics for relationship between nerve fibre densities (PGP 9.5) and nerve fibre expression (NGF and Trk-A)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman’s rho</td>
</tr>
<tr>
<td></td>
<td>Stroma nerve fibre density</td>
</tr>
<tr>
<td>NGF</td>
<td>rs</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>rs</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Trk-A</td>
<td>rs</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>rs</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
</tbody>
</table>

From the statistical analysis in Table 5.5.6, there were statistically significant correlations between stroma nerve fibre density and stroma NGF expression (p < 0.05). This suggests that with the increase in nerve fibre density in the stroma of the tissues, the intensity of NGF expression increased. However, there were no statistically significant correlations seen between nerve fibre density expression and Trk-A expression.
CHAPTER 6: DISCUSSION

6.1 Introduction

This retrospective case study is aimed to explore relationships in the expression and density of nerve fibres in the endometrial functional layer and peritoneal lesions of adolescents with endometriosis. These relationships were investigated in different micro-anatomical zones; the stromal, peristromal, and glandular regions. It also examined how these interactions were affected by patient’s medical treatment, menstrual stage, severity of disease, and age. Traditionally, studies have shown that different types of nerve fibres innervate peritoneal lesions of women with endometriosis (Tokushige et al. 2006b). Subsequently, other studies arose, showing the expression of nerve fibres in the endometrial functional layer of women with endometriosis (Tokushige et al. 2007). Collectively, further studies have suggested that these nerve fibres play a role in the pathophysiology of endometriosis associated pelvic pain (Bokor et al. 2009; Al-Jefout, Dezarnaulds, et al. 2009). However, this is the first time that a study has been done to investigate nerve fibres in the endometrial functional layer and peritoneal lesions specific to an adolescent age group.

Nerve fibres that are known to contribute to pelvic pain as seen in the eutopic endometrium and peritoneal lesions of older woman were also detected in our study of adolescent endometrial and peritoneal lesions. This study found that 18 (33.33%) out of 54 specimens had positively detected nerve fibres in the functional layer of the endometrium and 21 (70%) out of 30 peritoneal lesions had positively detected nerve fibres by using a pan neuronal marker Protein gene Product 9.5 (PGP 9.5). It was found that the average pan-neuronal density for adolescent
endometrial tissue was 57.70 mm$^2$ (range 5.33mm$^2$ to 209.52mm$^2$). It was also found that the stromal nerve fibre density was higher than that in the peristromal region for the adolescent lesion samples. On the contrary, Nerve Growth Factor (NGF) and its high affinity growth factor Tyrosine kinase – A (Trk-A) levels were found to be greatest at the glandular level compared to the stromal region in both the endometrial and peritoneal lesion tissues. It is important to note that for all histochemical runs, the positive controls consistently showed nerve fibre expression, confirming that the antibodies used and the laboratory protocols were not responsible for the variable staining results in the tissue samples for this study.

These immunohistochemical results were comparatively analysed with clinical results that had been retrospectively gathered from patients at the time of laparoscopic biopsy. This study showed that there were no statistically significant correlations between the expression of neuronal markers and nerve fibre densities with medical treatment, stage of endometriosis (disease severity), menstrual stage, and patient’s age. Although there was no statistically significant correlation with nerve fibre expression/intensity levels and medical treatment, our study demonstrated that for each medical treatment category the number of patients with positive nerve fibre expression were very close to the number of patients without nerve fibre expression. This suggests that medical treatment may not have had an effect on the detection of nerve fibres in this population.

Taken together, the data shows that nerve fibres known to contribute to pelvic pain in older woman with endometriosis are also expressed in adolescent functional endometrial and
peritoneal lesion tissue and that oral contraceptives/combined hormonal therapy did not show a reduced number of nerve fibres.

6.2 Neurogenesis

The results of the study revealed interesting findings regarding the distribution of nerve fibre densities, NGF and Trk-A expressions in the glandular and stromal regions of adolescent tissue samples. Previous studies have demonstrated multiple small unmyelinated nerve fibres in the eutopic endometrium and peritoneal lesions of women with laparoscopically confirmed endometriosis and none in the endometrium of women without the disease (Tokushige et al. 2006a; Tokushige et al. 2007). Although another research study in Belgium also demonstrated the expression of nerve fibres in endometriotic tissue (18 in 20) by PGP 9.5, they also found that patients without endometriosis in their study had positively stained nerve fibre expression as well (Bokor et al. 2009). Our study demonstrated that these small unmyelinated nerve fibres were also seen in adolescent endometrial tissue (18 of 54) and adolescent peritoneal lesions (21 of 30).

6.2.1 Neurogenesis in adolescent endometrial tissue samples

While 36 of 54 endometrial samples failed to express nerve fibres, this is not likely due to immunohistochemical staining but may be caused by sample quality itself. As we are aware, the human endometrium is a dynamic tissue, undergoing cyclical growth, functional change, and regression as part of the menstrual cycle. The functional endometrial layer varies in thickness during the menstrual cycle and is greatly responsive to hormones. Our adolescent endometrial specimens only sampled a narrow portion of the endometrium at a specific time. Bokor et al. (2009), indicated that differences in nerve fibre densities may
be attributed to nerve fibres not being homogeneously distributed throughout the endometrium and that not all areas are richly innervated (Bokor et al. 2009). Thus, perhaps the reason why nerve fibres in the endometrial tissues went undetected in some of our adolescent samples may be due to the fact that the sampled section itself may not have been innervated.

Upon investigation of tissues positive for nerve fibre expression, the mean nerve fibre density seen in the functional endometrium was 57.70mm$^2$ with a wide range of 5.33mm$^2$ to 209.52mm$^2$. Within the lesion tissue samples, the stromal median density of nerve fibres were shown as the distribution was quite varied with a median density of 4.00 mm$^2$ (IQR= 18.50) and in the peristroma the mean was 90.11mm$^2$ (SD = 130.81). Thus, revealing a higher nerve fibre density in the stroma than in the peristroma region.

In this study, NGF expression has been demonstrated in 68.52 % (37 of 54) of eutopic endometrial samples and Trk-A expression levels appeared in less of these samples with 42.59% (23 of 54). Our study also showed that there was a positive correlation between the NGF (p = 0.10) and Trk-A (p<0.001) glandular and stromal expression in eutopic endometrial samples, where both intensity levels were higher in the glands than in the stroma, although NGF and Trk-A expression were not consistently shown across all endometrial tissues. Previous studies have shown that there is an over expression of NGF, and an expression of Trk-A in the eutopic endometrium of women with endometriosis when compared with eutopic endometrium of women without the disease (Al-Jefout, Dezarnaulds, et al. 2009; Li et al. 2010). However, more recently, another publication
controversially has implicated that eutopic endometrium from women with endometriosis
does not exhibit neurotrophic properties (Barcena de Arellano et al. 2012), these are more
similar to our findings in the adolescent group. Barcena et al. (2012) reported that there
were no significant differences between the expression of NGF and Trk-A in eutopic
endometrial tissue of patients with endometriosis and those without the disease using
Western blot techniques (p>0.05) (Barcena de Arellano et al. 2012).

6.2.2 Neurogenesis in adolescent peritoneal lesion tissue

This study also demonstrated interesting findings for nerve fibre density and expression in
the adolescent peritoneal lesion samples. As mentioned previously, most (21 of 30)
adolescent peritoneal lesions showed nerve fibre expression. Remarkably, the pan neuronal
densities were significantly skewed in the stromal region of the lesion whereas it was
normally distributed in the peristroma region. There was a significant positive correlation
between stroma and peristroma densities (p <0.05), suggesting that the nerve fibre density
in the stroma was higher in than the peristromal region. This finding extends previous
work done in older women with endometriosis which demonstrated that lesions had
different micro-anatomical zones with greater nerve fibre density in the stroma (Tokushige
et al. 2006b). This outcome also illustrates what was observed in another study that most
nerve fibres were found in the stroma but most of these nerve fibres were also stained
positive with GAP-43 (growth associated protein -43); a marker used to show nerve
toutgrowth and regeneration (Mechsner et al. 2007). Together, these findings suggest
greater neurogenesis in the stroma for peritoneal lesions.
Our study showed that NGF was expressed in 50% (15 of 30) of the adolescent peritoneal tissue samples and similarly to the endometrial tissue, Trk-A were expressed in less of the samples with 33.33% (10 of 30) peritoneal lesion samples. NGF and Trk-A were shown to have a significant positive correlation between the gland and stroma intensities (NGF and Trk-A p values < 0.05). This trend suggests similar activity to the endometrial tissue, where the glandular expression is of higher intensity than the stromal region in both NGF and Trk-A manifestations. This result expands the work done by other studies showing greater NGF and Trk-A expression in glands compared to other histological zones of the lesion (Anaf et al. 2002; Tokushige et al. 2006b).

6.2.3 Nerve fibre density and nerve fibre expression

Our study explored relationships between the nerve fibre densities by PGP 9.5 and nerve fibres expression by NGF and Trk-A in all the adolescent endometrial tissues. Interestingly, upon analysis, there was a statistically significant correlation between stroma nerve fibre density and stroma NGF expression (p < 0.05), however, there was no correlation seen between nerve fibre density and Trk-A expression (p > 0.05). Our study demonstrated that with an increase in nerve fibre density in the stroma detected by PGP 9.5, the expression of NGF in the stroma correlated. This further promotes previous studies where patients with endometriosis express nerve fibres and NGF in functional endometrial tissues and peritoneal lesions (Tokushige et al. 2006b; Tokushige et al. 2007).
6.3 Nerve fibre expression in adolescents and medical treatment

Previous studies have suggested that hormonal treatment significantly reduces nerve fibre density in endometrium and endometrium and myometrium in women with endometriosis (Tokushige et al. 2008). Following that study, the effect of progestogens and combined oral contraceptives on nerve fibres densities and expression were explored and it was found that these medical treatments reduced nerve fibre density and NGF in peritoneal endometriotic lesions (Tokushige et al. 2009). Our study had 62 tissue samples that were on either combined oral contraceptive pill (COCP), Depo-Provera (Depo), non-steroidal anti-inflammatory drugs (NSAIDs), or Primolut and 22 were on none. The analysis with medical treatment was done as a whole, including all 84 endometrial and peritoneal lesion samples. For PGP 9.5, our investigations reported that there was not a significant difference between the expression of nerve fibres in our adolescent tissue samples on medication and those that were not. For patients that were not on any medications, 40.9% were positive for nerve fibre expression whilst 59.1% were negative. For patients on COCP, Depo, NSAIDs, and Primolut, 46.78% showed nerve fibre expression, while 53.22% did not show any. This result did not further extend what previous studies have shown. In review of the retrospectively collected data, medical treatment detail including length of time the patient had been taking the treatment to when/if it was ceased prior to biopsy was not collected. Therefore, with more details, combined with defined patient criteria, a larger sample size and a control group for comparison may clarify this finding.
6.4 The effects of severity of disease on nerve fibre expression in adolescents with endometriosis

Previously, women with endometriosis are thought to have a heightened level of pain perception compared to women without the disease. These studies have implied that an increase in nerve fibre density correlates with a higher level of pain in women with endometriosis (Asante & Taylor 2011). However, there is still controversy among studies as to pain symptoms correlating with severity of disease, with some suggesting that no correlation exists (Porpora et al. 1999) while others suggest a linear relationship between pain symptoms and severity of disease (Mechsner et al. 2009; Mechsner et al. 2010; Vincent et al. 2010).

In our study, at the time of laparoscopy endometriosis was staged based on American Society for Reproductive Medicine (ASRM) classification of endometriosis. As mentioned in previous chapters, the disease was classified into four stages (I-minimal, II- mild, III-moderate, IV-severe), however in this study we used a scale that incorporates 0; which is representative of no staging. Our reports show that 30 patients did not have an endometriosis stage, 13 patients were marked with endometriosis stage I, 12 had endometriosis stage II, 4 had endometriosis stage III, and none of the adolescent patients had endometriosis stage IV. Upon analysis, our study did not show any correlations between nerve fibre densities/expression and severity of disease based on the clinical staging of endometriosis for the adolescent samples. As mentioned previously, not all tissues showed nerve fibre expression, unlike previous research (Al-Jefout et al., 2009). Therefore we cannot provide further evidence to support previous findings of a relationship between severity of disease and nerve fibre densities.
6.5 The effects of menstrual stage and age on nerve fibre expression in adolescents with endometriosis

Earlier investigations of nerve fibre densities and expression in women with endometriosis have found no significant differences between nerve fibre density and different menstrual stages (Al-Jefout, Dezarnaulds, et al. 2009). Although our study has adolescents in various stages of the menstrual cycle; 23 patients in the secretory phase, 27 patients in the proliferative phase, 1 menstruating, and 8 patients with unknown menstrual stage, we did not find any correlations between nerve fibre densities/expression and menstrual stage (PGP 9.5, NGF, Trk-A, p value > 0.05). This further strengthens the previous outcomes of no relationship between nerve fibre density and specific menstrual stage.

Previous studies of nerve fibre expression in endometriosis are primarily reported in women of a reproductive age with most cases aged around 25 to 30 years old. Endometriosis although rare has been reported premenarchal girls as young as 11 years of age (Gogacz et al. 2012), and postmenopausal women (Manero et al. 2009). However, in these studies there have been no associations found with nerve fibre expression related to specific age groups. The sample size for our study was small, with 59 adolescent patients aged 15 to 19 years. The median age for this group was 18 years, with a small range of 4. Our study did not find any correlations between nerve fibre expression and patient’s age in this group. Although we did not find a nerve fibre correlation, when looking at the presentation of ages, there are more patients in the higher range, 18 and 19 years old, with only 2 patients at the lower end of the range; 15 years old. This could possibly be due to the fact that although the sole cause of endometriosis is unknown, the retrograde menstrual theory remains the most accepted, suggesting that endometriosis is caused
by menstrual flow. Thus, there are more patients with endometriosis in this study that are in the higher age group, as they most likely have already had their menses.

6.6 Future Directions

From our study, nerve fibres were detected in the functional endometrium and peritoneal lesions samples taken from adolescents with endometriosis who underwent dilatation and curettage for the investigation of pelvic pain. Although this study detected that 33.33% of endometrial tissue samples and 70% of peritoneal lesions had nerve fibre expression, the nerve fibre densities did not show correlations with any of the clinical characteristics (stage of endometriosis, menstrual stage and age) that were taken at time of laparoscopy. These findings substantiate to some extent that nerve fibre expression in the functional endometrium and peritoneal lesions of adolescents with endometriosis behaves similarly to what has been reported in older women with this disease. Our findings may contribute to subsequent studies.

For subsequent studies, rather than working with archival samples where patient information and tissue characteristics were incomplete, it would be best to prospectively collect well characterised endometriotic tissue ensuring good quality samples with completed clinical pathology.

Our study was a case study, where our results are mostly descriptive, to get a better analysis of neurogenesis in endometrial and peritoneal lesions, further analysis with a control group (adolescents without endometriosis) to compare would provide a better understanding of how nerve fibres present in endometriotic tissue.
As these patients underwent laparoscopy for the investigation of pelvic pain, for future adolescent studies, it would be good to collect pain scores to correlate against nerve fibre densities as nerves fibres have been suspected to be a cause of pain transmission for patients with endometriosis.

6.7 Conclusions

Despite the growing awareness of endometriosis, not only is there limited research about the disease specific to the adolescent population but little is known about the pain generation in this group. Traditionally older women have been studied as they are the majority of patients presenting with the disease. Chronic pelvic pain and endometriosis in adolescents can be debilitating. It is important to diagnose endometriosis in this age group so that appropriate treatment can be instituted for pain relief to prevent progression of the disease and to preserve fertility. Nerve fibre detection through biopsies has become increasingly popular in the field of endometriosis as it has been suggested as a less invasive method for diagnosis of the disease. As patients go undiagnosed for years, this method would be particularly beneficial to adolescents who do not undergo laparoscopy for histological confirmation of endometriosis due to its invasiveness, improving earlier diagnosis.

To summarise, although we had interesting correlations and findings in nerve fibre densities and expression, we did not find correlations between neurotrophic properties and clinical characteristics of patients. Although our study demonstrated nerve fibres previously seen in women with endometriosis in the functional adolescent endometrial tissue and peritoneal lesions, the percentage of nerve fibres, NGF and Trk-A expression was lower than other studies. As they
were not expressed in the majority of samples, particularly in the endometrial samples, we cannot be assured that our findings are a good representation of adolescents with endometriosis. Further detailed studies with a larger sample size, improved group criteria, experienced collection of samples, and a control group, are needed for a better understanding of nerve fibre expression in adolescent endometriosis.
APPENDICES

Appendix 1: Copy of ethics approval letter

27 July 2011

TO WHOM IT MAY CONCERN

This is to confirm that the RWH Research Committee and RWH Human Research Ethics Committee have considered the proposed study “Presence of endometrial nerve fibres in young women with pelvic pain” to be undertaken by Dr H Kaur, Professor M Hickey and Professor I Fraser.

The Committees are of the view that this study meets the National Health and Medical Research Council requirements for quality assurance / audit projects and will be endorsed as such by the RWH Research Committee and RWH Human Research Ethics Committee.

Mr A C B Hui
Administrative Officer
Research and Ethics Secretariat
REFERENCES


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