UTERINE BRAIN-DERIVED NEUROTROPHIC FACTOR AND ENDOMETRIOSIS

UTERINE BRAIN-DERIVED NEUROTROPHIC FACTOR AND ENDOMETRIOSIS

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Lay Abstract

Endometriosis is a chronic condition that affects over 10% of women of reproductive age. Women with endometriosis suffer from debilitating pelvic pain and it takes approximately 12 years before they are diagnosed during surgery. This is in part because there is no blood test to diagnose disease. We are interested in using a protein called brain-derived neurotrophic factor (BDNF) that is linked to several of the pathways that are disturbed in women with endometriosis as a means of determining whether or not a woman has endometriosis. The key goals of this thesis are to show that BDNF is a protein which is found in the uterus of many species, that it is controlled by estrogen, and that it might be useful in diagnosing endometriosis and monitoring how well a patient is responding to endometriosis treatment.

Abstract

Endometriosis is a chronic estrogen-dependent gynecological disease where endometrial cells implant at inappropriate sites causing significant pelvic pain, decreased quality of life, and often infertility. It affects 10% of women of reproductive age, and there is no minimally invasive diagnostic test. Consequently the time to diagnosis, which occurs during laparoscopic surgery followed by pathological confirmation of disease, is prolonged and exceeds 11 years. During this time, the disease often worsens and women thus experience avoidable morbidity. Additionally, endometriosis is a financial burden on the healthcare system; its annual cost was $69.4 billion (U.S.) and $1.8 billion (Canada) in 2009. For these reasons, identifying a clinical marker remains a top priority. Although multiple putative markers have been identified and reviewed, emerging evidence suggests a relationship between neurotrophins and endometriosis. The neurotrophins are growth factors recognized for promoting neuronal differentiation, growth, and maintenance. Recently, they have been shown to induce pathways central to endometriosis including proliferation, adhesion, angiogenesis and resistance to apoptosis, in cultured neurons, epithelial cells, fibroblasts, and cancer cell lines. Although two studies have suggested elevated concentrations of brain-derived neurotrophic factor (BDNF) in the plasma and eutopic endometrium of women with endometriosis, relatively little is known about uterine BDNF. Herein, we demonstrate the conservation of BDNF and its high affinity receptor in the mammalian uterus, and show the upregulation of BDNF and its low affinity receptor by estradiol in the mouse uterus. Encouraged by our results, we assessed circulating BDNF for its ability to differentiate between women with and without endometriosis, as excess

estradiol in endometriotic lesions might increase BDNF in women with disease. Our results revealed that circulating BDNF concentrations were significantly higher in women with endometriosis, particularly those with Stage I and II disease compared to controls. Furthermore, women with endometriosis undergoing ovarian suppression had significantly lower circulating BDNF than women not undergoing treatment, suggesting that BDNF may provide an opportunity to monitor patient response to treatment. Taken together, the data herein advances our limited knowledge of uterine neurotrophins, and supports a link between BDNF and endometriosis. I therefore strongly suggest that BDNF is a useful clinical marker of endometriosis, and encourage additional research to determine its role in the pathophysiology of disease.

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-J

"Today you are you, that is truer than true. There is no one alive who is youer than you."

– Dr. Seuss

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List of Abbreviations

15-PGDH	15-hydroxyprostaglandin dehydrogenase
17βHSD1	17-ß-hydroxysteroid dehydrogenase type 1
17βHSD2	17-ß-hydroxysteroid dehydrogenase type 2
ANOVA	Analysis of variance
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
β-actin	Beta-actin
β-tubulin	Beta-tubulin
BMI	Body mass index
bp	base pair
BSA	Bovine serum albumin
C	Celsius
CA-125	Cancer antigen-125
cDNA	complementary DNA
COX	Cyclooxygenase enzymes
CRP	C-reactive protein
CT	Computerized tomography
CYP2C19	Cytochrome P450 subfamily C
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	Estradiol
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay

FasL	Fas ligand
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome-wide association studies
h	Hour
ICAMs	Intercellular adhesion molecules
IL-6	Interleukin 6
kDa	kilodalton
MAPK	ras-mitogen-activated protein kinase
mBDNF	mature BDNF
Met	Methionine
µg	Micrograms
min	Minutes
miRISC	miRNA-induced silencing complex
miRNAs	microRNAs
µl	Microlitres
mL	Millilitres
µm	Microns
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	messenger RNA
N, n	Sample size
NCBI	National Center for Biotechnology Information
ng	Nanograms
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor, also called p75 neurotrophin receptor
NK	Natural killer cells
NSAIDS	Nonsteroidal anti-inflammatory drugs

NT-3, NTF3	Neurotrophin-3
NT4/5, NTF4, NTF5	Neurotrophin-4/5
NTRK	Neurotrophic tyrosine kinase receptor, also called Trk
NTRK2	Neurotrophic tyrosine receptor kinase 2
OVX	Ovariectomy
P	Level of significance/probability value
P_4	Progesterone
$P450_{AROM}$	Aromatase
p75	Nerve growth factor receptor, now called NGFR
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
pg	Picograms
PGs	Prostaglandins
PI3K	phosphatidylinositol 3-kinase
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
QUADAS	Quality Assessment of Diagnostic Accuracy Studies
rAFS	revised American Fertility Society
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROC	Receiver operating characteristic curve
s	Seconds
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
SE, SEM	Standard error of measurement
SF-1	Steroidogenic factor 1

sFasL	soluble Fas ligand
siRNAs	small interfering RNAs
SORT1	Sortilin
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween 20
TGF- β1	Transforming growth factor β1
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha
Trk	Tyrosine receptor kinase, now called NTRK
U/mL	Units/mL
UTR	Untranslated region
V	Volts
VEGF	Vascular endothelial growth factor

Declaration of Academic Achievement

Chapter 2

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Chapter 1

1.1: General Introduction –Endometriosis

Endometriosis is a chronic, estrogen-dependent disease of unknown etiology characterized by the presence and proliferation of endometrial glands and stroma implanted outside the uterus (reviewed in Olive and Pritts, 2001; Rogers et al., 2009; Giudice, 2010). The most common anatomical locations of endometriosis include the peritoneum lining the pelvis and abdominal cavity, ovaries, bowel, and the posterior cul-de-sac (reviewed in Rogers et al., 2009; Giudice, 2010).

There are several clinical manifestations of endometriosis including superficial red, black, and white peritoneal lesions, superficial red ovarian lesions, ovarian endometriomas, recto-vaginal nodules, deep-infiltrating endometriosis that may extend to the bladder, ureter, and bowel, and fibrous pelvic adhesions (reviewed in Giudice, 2010). While clinically identified and treated as one disease, it has been postulated that peritoneal, ovarian, and deep-infiltrating endometriosis may in fact be three distinct entities (Nisolle and Donnez, 1997). Nevertheless, the clinical signs of disease are difficult to assess, making the diagnosis of endometriosis a challenge. At present, endometriosis is presumptively diagnosed as a result of patient symptomatology, with laparoscopic surgery followed by histopathological confirmation of disease serving as the gold standard for diagnosis.

There are four clinical stages of endometriosis: minimal (Stage I), mild (Stage II), moderate (Stage III), and severe (Stage IV), classified according to lesion location and extent of disease using the revised American Fertility Society (rAFS) guidelines (American Society for Reproductive Medicine 1996). However, lesion type, activity of disease, and severity of disease as it relates to pain are not considered by these classifications. Although it is considered to be a benign condition, endometriosis is a progressive disease that worsens over time. In Stage I and II disease superficial red lesions, thought to be the most physiologically active lesions are abundant, whereas in Stages III and IV white lesions, adhesions, and deep infiltrating disease predominate.

Endometriosis affects women from all ethnicities and social groups, causing debilitating pelvic pain, emotional suffering, and often infertility (Eskenazi and Warner, 1997; Cramer and Missmer, 2002; Nnoaham et al., 2011). Women with endometriosis generally experience pelvic pain which can be continuous or associated with menstruation, intercourse, defecation, and/or urination (reviewed in Olive and Pritts, 2001; Rogers et al., 2009; Giudice 2010). The pain associated with endometriosis can be a result of peritoneal inflammation, deeply infiltrating disease, or nerve growth into or surrounding endometriotic lesions (Anaf et al., 2002; Berkley et al., 2005; Mechsner et al., 2007; Mechsner et al., 2009; Wang et al., 2009a). Interestingly, there is no correlation between the extent or stage of disease and the severity of pain (Kennedy et al., 2005; Hsu et al., 2011).

Although 'invisible', endometriosis poses significant quality of life issues as the pain associated with this disease can be so severe that it interferes with employment and leisure activities. Women with endometriosis lose an average of 10.8 hours a week from work, mainly due to reduced effectiveness (Nnoaham et al., 2011; Rogers et al., 2013). Indeed, some women are unable to maintain full-time employment because workplaces are unwilling to accommodate or are unsympathetic to their needs (Gilmour et al., 2008). The pain of the disease and social stigma of infertility can also have damaging effects on social functioning, emotional health, relationships with healthcare providers, vitality, and employment (Jones et al., 2004; Simoens et al., 2012). Additionally, endometriosis can be psychologically scarring, affecting self-image, and leading to depression, feelings of guilt, powerlessness, isolation, and concern the disease will be inherited by daughters (Jones et al., 2004; Gilmour et al., 2008).

1.2: Epidemiology of Endometriosis

It is widely accepted that approximately 10-20% of women of reproductive age (Moen and Muus, 1991; Eskenazi and Warner, 1997) are affected by endometriosis, amounting to an estimated 176 million women world-wide (Adamson et al., 2010). However, the prevalence of this condition reaches 50% in women undergoing diagnostic laparoscopy (Hemmings et al., 2004), and is reported to be an incidental finding in 18-35% of women presenting for tubal ligation or hysterectomy (Hemmings et al., 2004). It is generally accepted that endometriosis is underdiagnosed (Nnoaham et al., 2011). Therefore, determination of an exact incidence or prevalence rate of endometriosis has been a challenge because women

generally do not know when the onset of disease occurred, and many women remain asymptomatic, unaware that they have this condition.

Endometriosis is more common amongst first-degree relatives (mother, sister, daughter), pointing towards a heritable genetic predisposition to disease (Simpson et al., 1980; Malinak et al., 1980; Stefansson et al., 2002; Kashima et al., 2004; Templeman et al., 2008; Matalliotakis et al., 2008). However, no single specific genetic locus has been identified as a risk factor for endometriosis. Thus, it seems likely that the heritable risk associated with endometriosis is either multi-genic or mutations in several key genes produce a similar disease phenotype.

Genome-wide association studies (GWAS) are becoming a more common method of assessing genetic risk of endometriosis across the genome (Adachi et al., 2010; Painter et al., 2011; Nyholt et al., 2012; Albertsen et al., 2013), and a large sample size (thousands of women) for these types of studies is achieved through international collaborations (Near et al., 2011). Fortunately, several of the genetic risk loci identified in prior studies have been associated with endometriosis risk in GWAS. A polymorphism in the progesterone receptor (PR) that reduces the expression of PR-A, altering PR-A:PR-B ratio was shown to be associated with increased risk of endometriosis in a large scale study (Near et al., 2011), as was a region close to the cytochrome P450 subfamily C (CYP2C19) gene which was replicable in independent samples (Painter et al., 2011; Painter et al., 2014). Additionally, GWAS studies have identified several weakly associated SNPs, demonstrating a polygenic

risk for endometriosis that was replicated in an external population (Nyholt et al., 2012). The results of the study showed consistency across other GWAS studies, and 6 of the 9 identified loci (7p15.2, WNT4, VEZT, CDKN2B-AS1, ID4 and GREB1) were in or near genes known to be of biological relevance in endometriosis including uterine development, cellular growth in general, and carcinogenesis (Rahmioglu et al., 2014a).

At present, there is no single genetic risk factor for endometriosis, but rather evidence of its polygenic and multifactorial nature. Functional studies showing the effects of alteration of genes conferring genetic risk for endometriosis are required to elucidate their effect on biological pathways. The lack of readily identifiable genetic loci that are consistently associated with endometriosis risk has led to the recent consideration of epigenetic modifications (reviewed in Guo 2009), miRNAs (reviewed in Teague et al., 2010), and mitochondrial DNA (Cho et al., 2012; Govatati et al., 2012) as risk factors.

1.3: Etiology of Endometriosis

Although the specific cause(s) of endometriosis remains unknown, several theories explaining its pathogenesis have been put forward. Sampson's theory of retrograde menstruation which proposes that endometrial cells and tissue shed at menses are regurgitated into the peritoneal cavity via the fallopian tubes (Sampson, 1927) is the most widely accepted theory of disease pathogenesis. Retrograde menstruation affords endometrial cells access to the peritoneal cavity where they can adhere, implant, proliferate, survive, induce angiogenesis, produce immune modulators, and resist apoptosis.

Practically, the anatomical distribution of lesions supports this theory; implants tend to accumulate in the posterior pelvis, perhaps a result of gravity, and asymmetrically on the left side, perhaps due to the sigmoid colon acting as a barrier of fluid flow (Dmowski and Radwanska, 1984; Al-Fozan and Tulandi, 2003; Chapron et al., 2006; Kissler et al., 2011). However, Sampson's theory is not sufficient in explaining the pathogenesis of endometriosis because retrograde menstruation occurs in up to 90% of women (Koninckx et al., 1980; Blumenkrantz et al., 1981; Kruitwagen et al., 1991a; Kruitwagen et al., 1991b; Koninckx, 1994), yet the prevalence of endometriosis remains roughly 10% (Moen and Muus, 1991; Eskenazi and Warner, 1997). Therefore, factors other than just access to the peritoneal cavity must be considered. Additionally, Sampson's theory cannot explain the occurrence of endometriosis in women with uterine dysgeneis or agenesis (Rokitansky-Kuster-Hauser syndrome) (Rosenfeld and Lecher, 1981; Elliott et al., 2011; Troncon et al., 2014), nor its occurrence in organs outside of the peritoneal cavity including the brain (Ichida et al., 1993; Vilos et al., 2011), lung (Lattes et al., 1956; Rodman and Jones, 1962; Sevinc et al., 2013; Azizad-Pinto and Clarke, 2014), kidneys (Gauperaa and Stalsberg, 1977; Hellberg et al., 1991; Gupta et al., 2005; Dirim et al., 2009), and surgical scars in the skin (Schmitz and Grossbard, 1948; Martins, 1957; Chatziparadeisi et al., 2014). Sampson's theory is certainly insufficient in explaining the presence of endometriosis in men (Oliker and Harris, 1971; Martin and Hauck, 1985; Fukunaga, 2012; Gonzalez et al., 2014). Thus, alternate hypotheses of the etiology of endometriosis have been described including the vascular and lymphatic transport of endometrial fragments (Ueki, 1991), iatrogenic transplantation of endometrial fragments during surgery (Szlachter et al., 1980;

Sepilian et al., 2003), Müllerian remnant differentiation (Ugur et al., 1995), coelomic metaplasia (Matsuura et al., 1999), and most recently differentiation of bone marrow derived stem cells (Du and Taylor, 2007; Sasson and Taylor, 2008; Figueira et al., 2011), and menstrual blood stem cells (Nikoo et al., 2014). Each of these hypotheses have led researchers to examine the role of many biochemical pathways in the pathogenesis of endometriosis.

In addition to the aforementioned hypotheses of the etiology of endometriosis, there are reports that genetic predisposition (Stefansson et al., 2002; Wenzl et al., 2003; Albertsen et al., 2013), immune dysfunction (Kyama et al., 2003), and exposure to toxicants (Bruner-Tran et al., 1999; Rier and Foster 2002; Foster and Agarwal 2002; Rier and Foster 2003; Anger and Foster 2008) also increase the risk of endometriosis. However, even though risk factors have been identified and numerous biochemical differences in the peripheral circulation, peritoneal fluid, and endometrium have been documented between women with and without endometriosis (May et al., 2010; May et al., 2011; Fassbender et al., 2013), none are specific to endometriosis.

1.4: Pathophysiology of Endometriosis

The pathogenesis of endometriosis is complex and likely multifactorial (Figure 1). The physical access of endometrial cells to ectopic locations can be explained by theories proposing a uterine origin of disease (retrograde menstruation, vascular, lymphatic, iatrogenic transplantation, Müllerian remnant, menstrual blood stem cells) as well as those

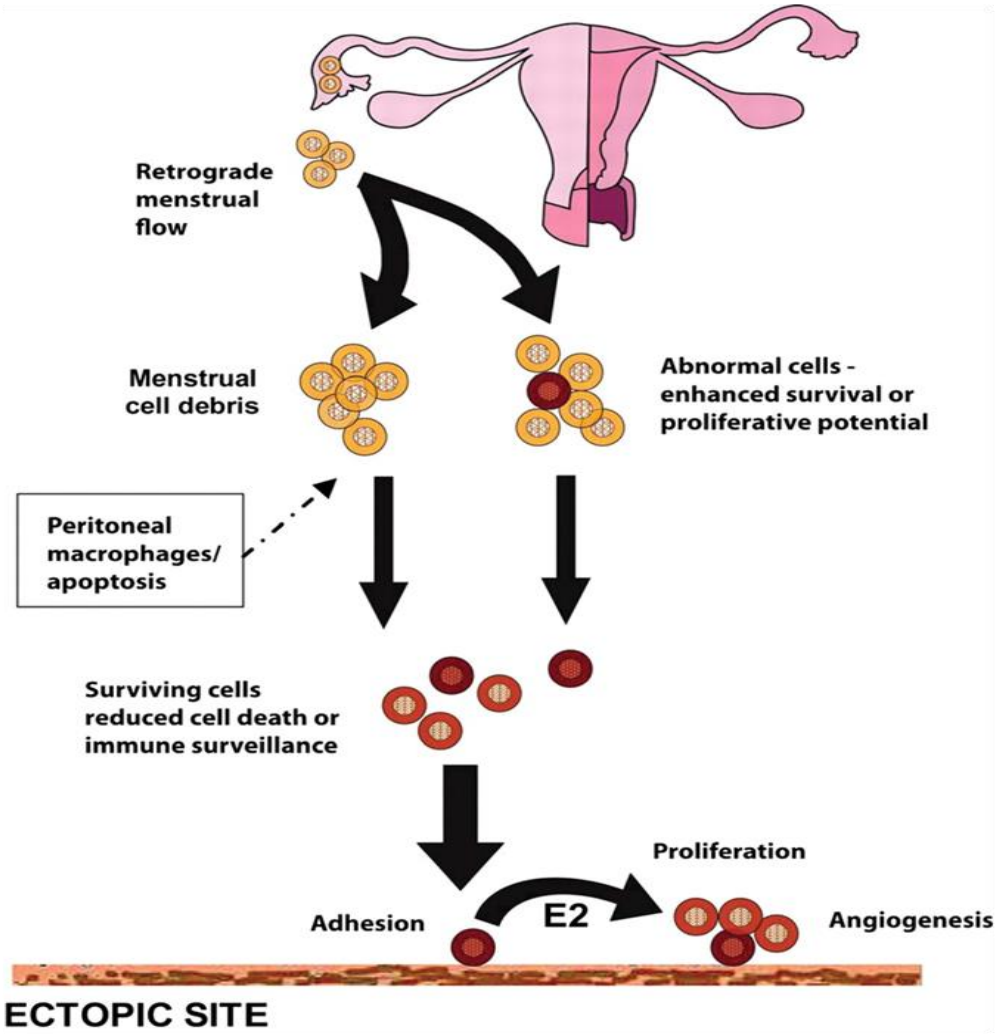

Figure 1: The Pathophysiology of Endometriosis.
A schematic representation of some of the possible mechanisms contributing to the development of endometriosis. Sampson's theory of retrograde menstruation suggests that endometrial cells are regurgitated into the peritoneal cavity each month during menses, and that these cells attach, invade, proliferate, evade the immune system, induce angiogenesis, and resist apoptosis in women with endometriosis. At ectopic sites the endometrial cells synthesize an excess of estradiol (E2) via their expression of aromatase, and are unable to respond to progesterone, rendering them progesterone insensitive. Other factors involved in the pathophysiology of endometriosis include a genetic predisposition towards disease, environmental, and lifestyle factors. This figure was reprinted from Montgomery et al., 2008, and used with permission from Oxford University Press.

proposing a non-uterine origin of disease (coelomic metaplasia, differentiation of bone marrow derived stem cells). As retrograde menstruation occurs in greater than 90% of women (Koninckx et al., 1980; Blumenkrantz et al., 1981; Kruitwagen et al., 1991a; Kruitwagen et al., 1991b; Koninckx, 1994), but only 10% are diagnosed with endometriosis, other properties, heritable or otherwise, must influence the propensity for the implantation and survival of endometrial cells in some women, but not others. In most women, the endometrial cells which are naturally refluxed into the peritoneal cavity each month are likely identified and removed by the immune system. However, in women prone to endometriosis the implanted cells persist, and have been demonstrated to activate pro-inflammatory factors including prostaglandins (Carli et al., 2009; Zhang et al., 2011; reviewed in Sacco et al., 2012; Rakhila et al., 2013), cytokines (Akoum et al., 1996; reviewed in Wu and Ho, 2003; Antsiferova et al., 2005; Kyama et al., 2006; Lin et al., 2014a), and chemokines (Akoum et al., 1995; Hornung et al., 1997; Bertschi et al., 2013; Franasiak et al., 2014; Leconte et al., 2014).

A reduction in innate immune activity has been found in women with endometriosis compared with those without. Natural killer (NK) cells were demonstrated to have reduced cytotoxicity in women with endometriosis, due to both a defect in NK activity and also due to endometrial resistance to NK cytotoxicity (Oosterlynck et al., 1991; Oosterlynck et al., 1992; Ho et al., 1995; Somigliana et al., 1996). Macrophages, phagocytic cells of the innate immune system, are also implicated in the pathogenesis of endometriosis. It is postulated that the peritoneal macrophages in women with endometriosis are overloaded with iron

from the refluxed menstrual effluent, resulting in oxidative stress and consequently inflammation (reviewed in Gupta et al., 2006). Indeed, iron-overloaded macrophages, called hemosiderin-laden macrophages, are one of the hallmark indicators of endometriosis (Zaatari et al., 1982). As opposed to NK cells, macrophages in women with endometriosis appear to have increased activation and may have differing phenotypes dependent upon their eutopic or ectopic localization (Halme et al., 1983; Halme et al., 1984; Halme et al., 1987; Kobayashi et al., 2012; Smith et al., 2012; Wang et al., 2013a; Cominelli et al., 2014; Takebayashi et al., 2015). Additionally, cells of the adaptive immune response may also contribute to the pathophysiology of disease (Dmowski et al., 1981; Gilmore et al., 1992; Witz et al., 1994; Ho et al., 1996; Ho et al., 1997; Antsiferova et al., 2005; Mier-Cabrera et al., 2011; Olkowska-Truchanowicz et al., 2013), mainly by promoting a pro-inflammatory environment.

When displaced endometrial fragments mainly comprised of epithelial and stromal cells encounter the mesothelial lining of the peritoneum it appears that endometrial stromal cells are responsible for initiating attachment of the endometriotic cells (Lucidi et al., 2005). The adhesion of patient-derived stromal cells to autologous peritoneal cells ranged from 10-45%, indicating that some women likely have factors that increase their odds of developing disease (Lucidi et al., 2005). Indeed studies have shown that epithelial and stromal cells in the endometriotic lesions have an enhanced ability to express adhesion molecules (Li et al., 2014) including integrins (Lessey et al., 1994; Witz et al., 2000; Klemmt et al., 2007), intercellular adhesion molecules (ICAMs) (Vigano et al., 1998; Lucidi et al., 2005; Pino et

al., 2009), laminin (Beliard et al., 1997; Locci et al., 2013), fibronectin (Beliard et al., 1997), and E-cadherin (Beliard et al., 1997), and to promote tissue remodelling and invasion by increasing expression of matrix metalloproteinases (MMPs), key enzymes responsible for extra-cellular matrix remodelling (Osteen et al., 1996; Bruner-Tran et al., 2002; Mulayim et al., 2004; Lucidi et al., 2005; Collette et al., 2006; Pino et al., 2009; Delbandi et al., 2013) and decreasing expression of tissue inhibitors of metalloproteinases (TIMPs) (Sillem et al., 2001; Chung et al., 2001; Chung et al., 2002; Protopapas et al., 2010).

In addition to adhering, attaching, and invading, the shed endometrial cells and tissue fragments entering the peritoneal cavity by retrograde menstruation must ultimately establish their own blood supply to grow and survive. Neovascularization, the formation of new blood vessels, at ectopic locations is one key factor in the development of endometriosis. Several studies have documented the expression of pro-angiogenic factors including vascular endothelial growth factor (VEGF) (Donnez et al., 1998; Tan et al., 2002; Bourlev et al., 2006; Machado et al, 2008; Di Carlo et al., 2009; Ramon et al., 2011), members of the fibroblast growth factor family (Wing et al., 2003), angiopoietins (Di Carlo et al., 2009; Gescher et al., 2004; Jingting et al., 2008), macrophage migration inhibitory factor (Yang et al., 2000), and stromal cell-derived factor 1 (SDF-1) (Furuya et al., 2007; Virani et al., 2013) in endometriotic lesions. Promotion of blood vessel development at ectopic sites appears to be the result of at least two angiogenic mechanisms working in concert. The menstrual endometrium of women with endometriosis inherently expresses an

abundance of pro-angiogenic cytokines which may lead to the classical activation of angiogenic pathways (Kyama et al., 2006). In addition, circulating endothelial progenitor cells are documented to account for 37% of the *de novo* formation of the ectopic microvessels (Laschke et al., 2011). Thus, both mechanisms are liable to promote lesion growth and proliferation at ectopic sites.

Further contributing to the development, growth, and survival of endometrial cells at ectopic locations is their ability to resist apoptosis. Endometriotic cells may inherently have increased expression or deregulation of anti-apoptotic regulators including B-cell lymphoma 2 (Bcl-2) (Watanabe et al., 1997; Jones et al., 1998; Meresman et al., 2000; Goumenou et al., 2001; Beliard et al., 2004), oncogene c-myc (Schenken et al., 1991; Schneider et al., 1998; Johnson et al., 2005; Meola et al., 2010; Pellegrini et al., 2012), and Fas ligand (FasL) (Selam et al., 2002), or downregulation of pro-apoptotic genes including Bcl-2-associated X protein (BAX) (Goumenou et al., 2001; Johnson et al., 2005), tumor suppressors (Braun et al., 2007; Laudanski et al., 2009; Zubor et al., 2009), and caspases (Braun et al., 2007) that serve to enhance their survival.

Considering that there are a multitude of biochemical factors that are reportedly dysregulated in women with endometriosis, its diagnosis should be simpler than it is currently. Even though cellular adhesion, invasion, proliferation, inflammation/immune dysfunction, angiogenesis, resistance to apoptosis, local estrogen biosynthesis, progesterone insensitivity, genetic predisposition, environment, and lifestyle all contribute

to the development and progression of endometriosis, current diagnosis remains dependent on invasive surgery and histopathology. As such, there are significant delays in patient diagnosis, and major costs associated with this disease.

1.5: Diagnostic Delays and the Cost of Endometriosis

At present, it is not possible to accurately diagnose the presence of endometriosis based on symptoms, clinical examination, imaging techniques (ultrasonography, magnetic resonance imaging (MRI), computerized tomography (CT)), blood test or urine test. This is in part because there are no validated diagnostic markers or panel of markers that are specific to endometriosis (May et al., 2010; May et al., 2011; Vodolazkaia et al., 2012). Although more than 100 putative peripheral biomarkers related to the pathways reported to be dysregulated in patients with endometriosis (adhesion, apoptosis, angiogenesis, hormonal, growth factor, and immunological) (reviewed in May et al., 2010) have been proposed and reviewed for their ability to provide a diagnostic test with a high sensitivity and specificity, none have proven reliable. As such, the length of time between a patient presenting with symptoms of endometriosis until confirmed diagnosis is 11.7 years in the U.S. (Ballard et al., 2006), and this statistic is likely similar in Canada.

Endometriosis is one of the largest national healthcare expenditures (Gao et al., 2006; Simoens et al., 2007; Simoens et al., 2012) with the annual cost being approximately $69.4 billion in the U.S. in 2009 (Simoens et al., 2012; reviewed in Burney and Giudice, 2012). In 2009 the annual cost of surgically confirmed cases in Canada was $1.8 billion (Levy et

al., 2011). The costs were dominated by indirect healthcare costs (mainly loss of productivity) accounting for 66% of the total, while direct costs (surgery, monitoring of disease, hospitalization, physician visits, medication) accounted for the remaining costs (Simoens et al., 2012). This is significantly more than comparable chronic conditions including Crohn's disease and migraines (Simoens et al., 2007), and is likely a result of the fact that the disease is poorly understood, difficult to diagnose, and progressively worsens over time (Koninckx et al., 1991; D'Hooghe and Debrock, 2002). Its chronic nature is an enormous burden on the healthcare system. Therefore there is an urgent and pressing need to identify a diagnostic marker of disease.

1.6: Diagnostic Markers

The symptoms of endometriosis are shared by other gynecological and gastrointestinal disorders and their non-specific nature makes endometriosis difficult to diagnose. Also, because no reliable diagnostic marker has been found, a simple blood or urine test to diagnose disease remains elusive. Thus, the gold-standard diagnostic for endometriosis remains visualization of endometriotic lesions during laparoscopy combined with histo-pathological confirmation of disease (Kennedy et al., 2005). Laparoscopic resection of endometriosis is a surgical procedure that is not without risk (Darai et al., 2007; Slack et al., 2007). Although the risks are rare, women with endometriosis can expect to have multiple surgeries over their lifetime (Jarrell, 2010). Thus, according to an international panel of endometriosis experts, the identification of a non-invasive diagnostic test for endometriosis is a top research priority (Rogers et al., 2009).

While no specific markers of endometriosis have been identified, measurable biological markers, biomarkers, that correlate with a specific outcome or disease state (Kingsmore, 2006) have been extensively reviewed (Figure 2) (May et al., 2010; May et al., 2011; Fassbender et al., 2013). Peripheral biomarkers of endometriosis were systematically reviewed by May et al., 2010 from high quality studies meeting their modified Quality Assessment of Diagnostic Accuracy Studies (QUADAS) (Whiting et al., 2003) criteria (Figure 3), and eutopic biomarkers were later reviewed under the same criteria (May et al., 2011). While changes in either endometrial or peripheral biomarkers can be used to diagnose disease, a non-invasive test would likely be preferred. Non-invasive tests for endometriosis would include tests performed on the peripheral blood, serum, plasma, urine, or menstrual effluent whereas semi-invasive tests would include tests performed on uterine curettages or peritoneal fluid collected by fine needle aspiration (Fassbender et al., 2013).

The vast majority of studies aimed at identifying biomarkers of endometriosis have concentrated on single factors known to be involved in disease pathogenesis and progression including inflammatory mediators, adhesion molecules, angiogenic regulators, growth factors, and enzymes of the estrogen biosynthesis pathway. However, recent studies have taken to identifying and evaluating panels of endometriosis biomarkers (Seeber et al., 2008; Mihalyi et al., 2010; Kyama et al., 2011; El-Kasti et al., 2011; Vodolazkaia et al., 2012; Borrelli et al., 2015). The combined use of putative markers is likely to enhance both the sensitivity and specificity of the test. The sensitivity of a test refers to the probability of

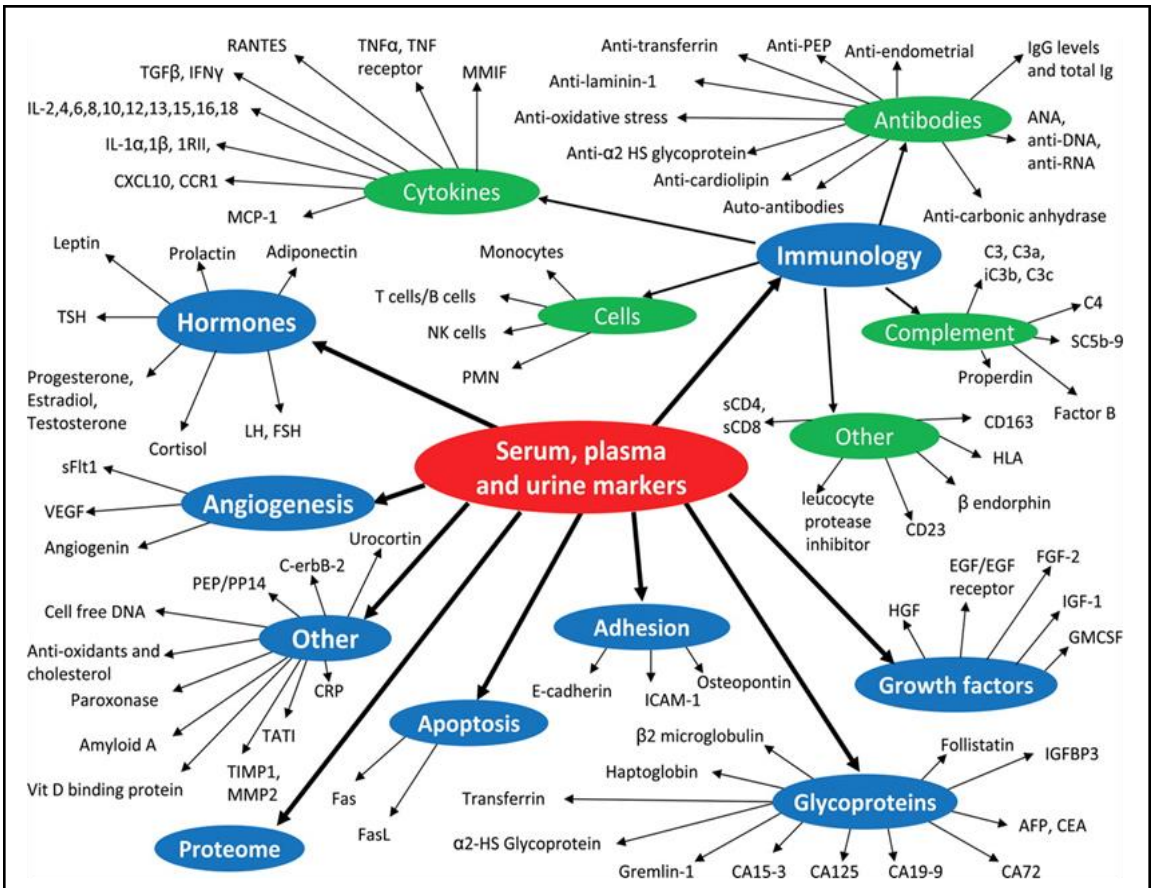

Figure 2: Putative Peripheral Biomarkers of Endometriosis.
A spider diagram depicting more than 100 putative biomarkers of endometriosis recently reviewed in a systematic review. Each of the putative markers has been assessed as a non-invasive biomarker for endometriosis and were quantified in either the serum, plasma, or urine. In the figure, the biomarkers are listed by pathway or family. Each of the pathways implicated in the pathophysiology of endometriosis have been heavily mined for biomarkers, and yet no specific marker or panel of markers has been identified. Reprinted from May et al., 2010, and used with permission from Oxford University Press.

Modified QUADAS criteria used for assessing studies.

Criteria	Yes	No	Unclear
1. Were patients and controls recruited from women with symptoms consistent with endometriosis?			
2. Were selection criteria clearly described? Did the study describe time frame, consecutive recruitment, inclusion/exclusion criteria?			
3. Was the time period between the diagnosis and biomarker test short enough to avoid a change in disease status?			
4. Were the methods for testing sufficiently explained?			
5. Were the biomarker test results interpreted in a blinded fashion?			
6. Was the diagnosis of endometriosis made without knowledge of the biomarker test results?			
7. Were uninterpretable/intermediate test results reported?			
8. Were withdrawals from the study explained?			
9. Were samples collected at a consistent phase of the cycle, or results corrected for cycle phase?			
10. Were samples collected from women with a particular stage(s) of disease, or results corrected for stage?			

Figure 3: Modified Quality Assessment of Diagnostic Accuracy Studies (QUADAS) Criteria. A list of the modified QUADAS criteria employed to systematically review the literature for peripheral (May et al., 2010) and endometrial (May et al., 2011) biomarkers of endometriosis. The QUADAS score is directly proportional to the quality of the study. Figure reprinted from May et al., 2011, with permission from Oxford University Press.

the test being positive when disease is present, and the specificity refers to the probability of the test being negative when disease is absent.

1.6.1: Immunological Biomarkers

Many immunological factors including immunoglobulins, cytokines, chemokines, and immune cell populations have been assessed for their ability to differentiate between women with and without endometriosis (reviewed in May et al., 2010; May et al., 2011). The most widely studied peripheral immune biomarkers include cytokines, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), which are potent pro-inflammatory cytokines. Both cytokines have been documented to be positively correlated with endometriosis in several studies (Matalliotakis et al., 1997; Pellicer et al., 1998; Bedaiwy et al., 2002; Pizzo et al., 2002; Darai et al., 2003; Iwabe et al., 2003; Xavier et al., 2006; Cho et al., 2007; Martinez et al., 2007; Othman et al., 2008). However, almost as many studies have failed to confirm these differences (Vercellini et al., 1993; Somigliana et al., 2004; Kalu et al., 2007; Jee et al., 2008; Seeber et al., 2008).

Although there is evidence of a dysfunctional immune response in women with endometriosis, research has failed to show a consistent change in immunological factors including total circulating antibodies, autoantibodies, T cells, B cells, NK cells, and macrophages (reviewed in May et al., 2010) in the peripheral fluids. C-reactive protein (CRP) is an acute phase protein that is used to monitor inflammatory processes. As such, it too has been tested for its ability to identify endometriosis. In an initial report, CRP was

found to be associated with endometriosis, in particular late stage disease (Abrao et al., 1997). However, several subsequent studies failed to identify systemic changes in CRP (Xavier et al., 2006; Kianpour et al., 2012; Thubert et al., 2014).

Other than cytokines and CRP, a recent systematic review highlighted the use of the three most studied chemokines (CXCL8, CCL2, and CCL5) as peripheral biomarkers of endometriosis (Borrelli et al., 2014). In the review, 40 of the 62 studies included (64%) found significantly increased CXCL8, CCL2, and/or CCL5, alone or in combination, in women with endometriosis compared with controls. However, the results were dependant on the fluid sampled (peripheral blood versus peritoneal fluid), and unfortunately the higher diagnostic value was usually associated with the peritoneal fluid, which is less readily accessible than blood (Borrelli et al., 2014). Although there was often a lack of agreement between studies as to which chemokines are increased in women with endometriosis, CXCL8 was significantly higher in the peritoneal fluid in 94% of the studies reviewed, and might be a useful addition to a panel of peritoneal fluid biomarkers. Unfortunately, the successful application of CXCL8 as a peripheral blood biomarker is unlikely, as 54% of the studies did not find elevated CXCL8 in the circulation of women with endometriosis (Borrelli et al., 2014).

1.6.2: Angiogenic Biomarkers

Of the many angiogenic factors shown to participate in the pathophysiology of endometriosis which were discussed in section 1.4, VEGF has been the most extensively studied for its ability to provide a robust peripheral marker of disease. However, many studies have failed to identify a strong link between elevated VEGF in the serum or urine and the presence of endometriosis (Pellicer et al., 1998; Gagne et al., 2003; Potlog-Nahari et al., 2004; Bourlev et al., 2006; Cho et al., 2007; Pupo-Nogueira et al., 2007; Othman et al., 2008). As a result, the use of VEGF as a biomarker of endometriosis is not defensible.

1.6.3: Apoptotic Biomarkers

Even though many apoptotic factors, discussed in section 1.4, are documented to be involved in the pathophysiology of endometriosis in some manner or another, very few have been assessed as peripheral biomarkers of disease (reviewed in May et al., 2010). A soluble form of FasL (sFasL), created as a result of FasL cleavage at the cell surface by matrix metalloproteinases, was quantified in the serum and was associated with Stage III and IV endometriosis (Garcia-Velasco et al., 2002). Another study found elevated sFasL to be significantly higher in women with endometriosis than fertile and infertile controls (Linghu et al., 2004). When cells expressing the FasL receptor (Fas) interact with FasL, they undergo apoptosis (reviewed in Lettau et al., 2011). Two studies have quantified circulating sFas in women with and without endometriosis, and equivalent concentrations were found by each study (Linghu et al., 2004; Kalu et al., 2007). Even though there is evidence supporting the involvement of many apoptotic factors in the pathophysiology of

endometriosis, most have not been assessed as biomarkers of disease, and thus none are currently used as such.

1.6.4: Tissue Remodelling Biomarkers

The involvement of tissue remodeling factors within the ectopic lesions in endometriosis is undeniable, and several of these factors have been quantified and assessed as peripheral biomarkers of endometriosis. ICAM-1 has had conflicting results as a biomarker for endometriosis with some studies reporting increased circulating concentrations in Stage I and II disease (Wu et al., 1998; Matalliotakis et al., 2001), others reporting elevated circulating levels associated with Stage III and IV (Daniel et al., 2000; Somigliana et al., 2002), and others finding no difference at all (De Placido et al., 1998) or conflicting results (Barrier and Sharpe-Timms, 2002). Soluble MMP-2 was found to be elevated (Huang et al., 2004), while TIMP-1 was found to be reduced (Sharpe-Timms et al., 1998) in women with endometriosis, corresponding to what is observed in the endometriotic lesions.

Of all of the putative biomarkers of endometriosis, cancer antigen-125 (CA-125) has been the most thoroughly studied and is the only marker occasionally used in clinical practice. CA-125 is a glycoprotein expressed on the surface of cells derived from the coelomic and Müllerian epithelium (endocervix, endometrium, fallopian tubes, peritoneum, pleura, pericardium), that can be cleaved into a soluble form and quantified in the peripheral circulation (reviewed in Spaczynski and Duleba, 2003). Although it has been employed for

over 20 years as a biomarker of endometriosis, it is more commonly used to diagnose and monitor ovarian cancer. Nonetheless, a meta-analysis published in 1998 suggested that CA-125 was a better biomarker of Stage III and IV endometriosis than Stage I and II (Mol et al., 1998). Studies published since continue to support a link between circulating CA-125 and endometriosis (Abrao et al., 1999; Somigliana et al., 2004; Agic et al., 2008; Seeber et al., 2008), and to describe a positive correlation between CA-125 and Stage III/IV disease (Chen et al., 1998; Amaral et al., 2006; Maiorana et al., 2007; Martinez et al., 2007; Rosa e Silva et al., 2007).

Despite its use as a putative endometriosis biomarker, CA-125 is not routinely employed as a diagnostic test for endometriosis. Perhaps due in part to its wide range of reported sensitivities (4-100%) (Mol et al., 1998). However, the use of CA-125 as a biomarker, particularly of Stage III/IV endometriosis, should not be abandoned. As with many studies describing putative biomarkers of endometriosis, most CA-125 studies suffer from a lack of standardization, making them difficult to compare. One study alluded to the effect of disease phenotype on CA-125 concentrations by demonstrating that the sensitivity of CA-125 as a biomarker of endometriosis was superior in women with endometriomas (79%) than without (44%), using the same arbitrary cut-point (Kitawaki et al., 2005). Therefore, while direct comparison of putative biomarkers reported in the past may prove difficult, the percentage of reports describing an association with endometriosis can certainly help to guide biomarker re-assessment and research. In the future, we must strive to standardize endometriosis biomarker studies, an aim which has been proposed by several recent reports,

in order to be able to compare putative markers between studies (Becker et al., 2014; Fassbender et al., 2014; Rahmioglu et al., 2014b; Vitonis et al., 2014).

1.6.5: Estrogen Dependence

Many biochemical differences have been reported between women with and without endometriosis, the most prominent of which is altered estrogen biosynthesis. Endometriotic lesion survival depends on estrogen which is acquired from the ovary, or synthesized from the conversion of androgens to estradiol (E_2) via aromatase ($P450_{AROM}$) (Figure 4) (Noble et al., 1996; Kitawaki et al., 1997; Fazleabas et al., 2003; Bukulmez et al., 2008a; Bukulmez et al., 2008b). Indeed, endometriosis is an estrogen dependent condition as its symptoms improve after surgical and natural menopause (reviewed in Kitawaki et al., 2002), or after medical therapies that suppress endogenous estrogens (Donnez et al., 1997; Fedele et al., 2004). In fact, the majority of medical therapies for endometriosis significantly suppress endogenous estrogen, clinically exploiting the reliance of the endometriotic lesions on estrogen for their growth and survival (reviewed in Giudice 2010). However, prolonged estrogen deprivation is not without side-effects, and should only be used as a short-term strategy. A hypoestrogenic state, even as short as 6 months in duration, increases the risk of developing osteoporosis, particularly in women who have not yet attained their peak bone mineral density (Agarwal, 2002).

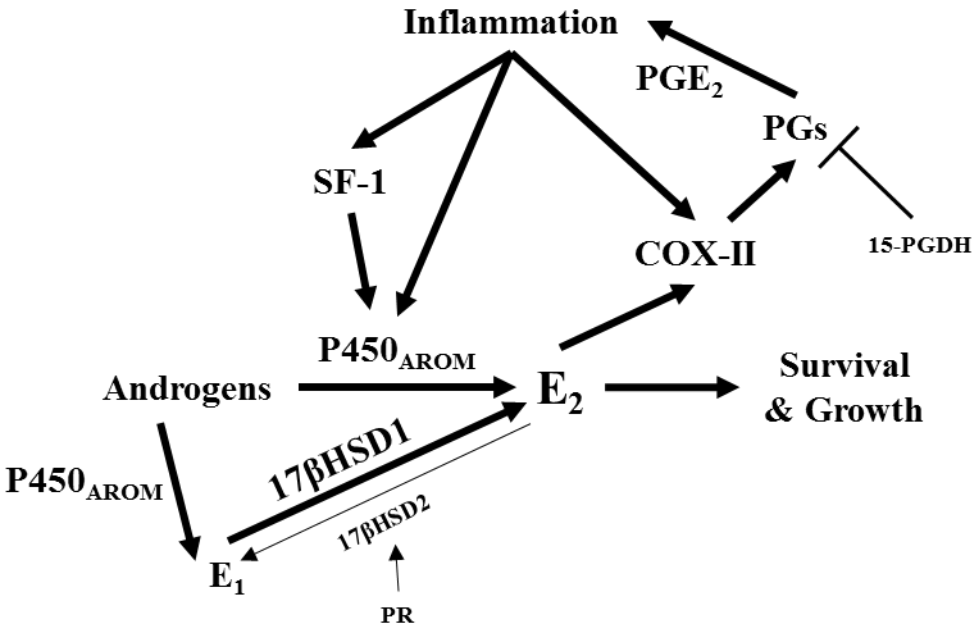

Figure 4: Excess Estrogen Synthesis in Ectopic Endometriotic Lesions. An abundance of estradiol (E_2) is synthesized in the endometriotic lesions through the conversion of androgens to estrogens via the aromatase enzyme ($P450_{AROM}$). E_2 induces the expression of cyclooxygenase II (COX-II) which increases the formation of prostaglandins (PG), including the potent inflammatory mediator PGE_2. The inflammatory microenvironment of the lesion is further exacerbated by inadequate quantities of the PG metabolizing enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH), and the positive feedback loop created between inflammation and E_2. Additionally, steroidogenic factor 1 (SF-1) a transcription factor for $P450_{AROM}$ is regulated by inflammatory factors. The excess E_2 in the ectopic lesions also arises from a reduced ability to metabolize E_2 to the less potent estrone (E_1) due to the decreased expression of 17-β-hydroxysteroid dehydrogenase type 2 (17βHSD2). It is postulated that progesterone insensitivity or inability to induce signalling through the progesterone receptor (PR) may be responsible for the downregulation of 17βHSD2. Finally, 17-β-hydroxysteroid dehydrogenase type 1 (17βHSD1) is increased in the endometriotic lesions, further promoting the synthesis of E_2. Combined, these factors heavily support the growth and survival of endometriotic lesions by increasing local E_2.

To circumvent this problem, many of the estrogen-depleting medical therapies are now accompanied by low-dose hormonal 'add-back' therapy (reviewed in Giudice 2010). This has proven effective in maintaining bone mineral density and managing endometriosis symptoms, without stimulating disease (Hornstein et al., 1998; Sagsveen et al., 2003). The estrogen threshold hypothesis supports the notion that low circulating concentrations of estradiol (30-45pg/mL), about ten times lower than physiological concentrations, is adequate to prevent bone loss but is not adequate to stimulate the growth and proliferation of endometriotic lesions (Barbieri, 1992). Thus, endometriosis is an estrogen dependent disease that requires concentrations of estrogen exceeding 15% of the circulating physiological levels.

1.6.5.1: Aromatase

Unfortunately, there is another source of estrogen other than the systemic circulation in women with endometriosis. Several groups have reportedly found the expression of $P450_{AROM}$ in the ectopic lesions in women with endometriosis (Noble et al., 1996; Kitawaki et al., 1997; Heilier et al., 2006; Matsuzaki et al., 2006a; Velasco et al., 2006; Bukulmez et al., 2008a; Bukulmez et al., 2008b). $P450_{AROM}$ is an enzyme in the estrogen biosynthesis pathway that ultimately helps convert androgens to E_2. This provides a mechanism through which the lesions are able to synthesize their own estrogen, thus promoting survival and growth. Additionally, the expression of $P450_{AROM}$ and its transcription factor, steroidogenic factor 1 (SF-1) are induced by inflammation (Attar et al., 2009), and pelvic inflammation

is another characteristic feature of endometriosis. Inflammatory mediators including prostaglandins (PGs) are formed by the cyclooxygenase enzymes (COX), and COX-II expression can be increased by E_2 (Tamura et al., 2004). As such, a positive feedback loop exists within the endometriotic lesions where the local synthesis of E_2 via $P450_{AROM}$ induces COX-II and PGE_2, which in turn increases SF-1 and $P450_{AROM}$ (Noble et al., 1997; Attar et al., 2009). Together, these factors contribute to an estrogen excess in the ectopic implants.

1.6.5.2: Estrogen Excess in Ectopic Implants

The E_2 excess resulting from the expression of $P450_{AROM}$ in the ectopic lesions is further exacerbated by the decreased expression of 17-ß-hydroxysteroid dehydrogenase type 2 (17βHSD2), an enzyme that catalyzes the conversion of E_2 to the less potent estrogen estrone (E_1), (Zeitoun et al., 1998; Matsuzaki et al., 2006a; Delvoux et al., 2009), and increased 17-ß-hydroxysteroid dehydrogenase type 1 (17βHSD1), the enzyme that converts E_1 to E_2 (Delvoux et al., 2009) in endometriotic lesions. Consequently, this leads to an excess of the most potent of the three estrogens, E_2, in the ectopic lesions. Indeed, E_2 concentrations in the ectopic lesions can be 2 to 6 times higher than circulating levels, and endometriosis treatments aimed at promoting a hypoestrogenic state have been shown to significantly decrease E_2 concentration in all lesion types (Huhtinen et al., 2012).

Even though the ectopic lesions have an excess of E_2, the circulating concentration of E_2 does not differ between women with and without endometriosis (Adamyan et al., 1993; Huhtinen et al., 2012), and cannot be used as a biomarker for endometriosis.

1.6.5.3: Prostaglandins

In addition to having increased quantities of inflammatory cytokines and chemokines, the peritoneal microenvironment in women with endometriosis has been shown to have increased concentrations of prostaglandins (PGs) (Badawy et al., 1984; De Leon et al., 1986; Wu et al., 2002). Furthermore, PG are synthesized by the endometriotic lesions, with increased PG production in the implants from patients with mild to moderate disease (Vernon et al., 1986). Rather than relating to the stage of disease, PG synthesis is dependent on lesion type and activity, with red lesions (active disease) producing more than twice the PGs as brown or black lesions (less active disease) (Vernon et al., 1986; Lousse et al., 2010). PGs likely contribute to the pathophysiology of endometriosis by directly mediating the inflammatory, and pain pathways, and by indirectly mediating proliferation via the upregulation of SF-1, $P450_{AROM}$ and thus E_2.

Prostaglandins are potent inflammatory mediators synthesized from membrane phospholipids or diacyl-glycerol via their intermediary conversion to arachidonic acid (reviewed in Lousse et al., 2012). Arachidonic acid is subsequently converted to prostaglandins by the cyclooxygenase enzymes (COX) and PG synthases. Both families of enzymes are markedly increased in the ectopic lesions of women with endometriosis

particularly in active lesions (Lousse et al., 2010; Rakhila et al., 2013). Furthermore, 78.5% of ovarian endometriomas, 13.3% of recto-vaginal nodules, and 11.1% of peritoneal implants have been found to express COX-II, an inducible form of the COX enzyme (Fagotti et al., 2004). Stimulation of COX-II likely occurs as a result of the inflammation associated with endometriosis, as COX-II expression can be driven by high levels of pro-inflammatory cytokines (Lindstrom and Bennett, 2004). The expression of PG synthesis enzymes is not limited to the endometriotic lesions. In addition, the peritoneal macrophages isolated from women with endometriosis express more COX-II (Lousse et al., 2010), and release more PGs, including $PGF_{2\alpha}$, and PGE_2, the most biologically active form (Wu et al., 2002) than women without endometriosis. PGs, including PGE_2, are metabolized to biologically inactive form by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), and two studies have quantified 15-PGDH in ectopic endometriotic lesions. Although one study failed to find a change in 15-PGDH (Rakhila et al., 2013), and the other found elevated 15-PGDH transcripts in peritoneal lesions which the authors postulated was an insufficient attempt to guard against PG overproduction (Lousse et al., 2010), they highlight the imbalance between the synthesis and degradation of PGs.

The abundance of PGs in endometriotic lesions is bound to increase PG signalling via their receptors. Indeed, three of the PGE_2 receptors (EP1, EP2, and EP4) are expressed by a transformed and passaged cell line derived from an endometriotic lesion (Banu et al., 2009), with EP2 and EP4 being the most dominant forms. The inhibition of EP2 and EP4 in the same cells, *in vitro*, has been shown to inhibit proliferation (Lee et al., 2010), decrease

migration and invasion (Lee et al., 2011), decrease adhesion (Lee et al. 2013), and increase apoptosis (Banu et al., 2009) in endometriotic epithelial and stromal cells. Thus, both EP2 and EP4 are likely involved in many of the central pathways associated with the pathophysiology of endometriosis.

While PGs are integral mediators of inflammation and thought to be involved in endometriosis pathogenesis, few studies have quantified PGs in the circulation. One study found elevated levels of PGE_2 in the peritoneal fluid and serum of women with endometriosis, and reported a positive correlation with disease stage (Li et al., 2005). Another study reported elevated $PGF_{2\alpha}$ in the urine and peritoneal fluid of women with disease (Sharma et al., 2010). However, other studies failed to find a difference in peritoneal fluid or circulating PGs (Rock et al. 1982; Sgarlata et al., 1983). The contradictory results might be explained in part by the improved assay sensitivity in recent studies, the short half-life of PGs (Ishihara et al., 1991; reviewed in Lousse et al., 2012), or the fact that almost every cell type can synthesize PGs, and thus they are not specific to endometriosis, but rather associated with a variety of inflammatory conditions (reviewed in Lousse et al., 2012). For the aforementioned reasons, a circulating PG biomarker for endometriosis seems unlikely.

1.6.6: Progesterone Resistance

One of the functions of progesterone in the endometrium is to oppose the mitogenic actions of estrogen. It induces the differentiation and limits growth of the endometrial epithelial and stromal cells (reviewed in Bulun et al., 2006). However, in endometriotic lesions, which are exposed to elevated concentrations of E_2 that contributes to lesion survival, there is increasing evidence to suggest that the ectopic lesions are unable to respond to progesterone (reviewed in Bulun et al., 2006). Perhaps a result of the endometriotic lesions having attenuated PR expression when they were compared to matched eutopic endometrium, or because they are lacking PR-B expression (Attia et al., 2000). Even the eutopic endometrium in women with endometriosis has been found to have an incomplete transition to progesterone responsiveness, and is postulated to enhance the survival and implantation of the regurgitated endometrial cells (Burney et al., 2007).

The mechanism by which progesterone resistance is thought to occur in endometriotic lesions is that due to low levels of PR-A, and/or the absence of PR-B, the endometrial epithelial cells present in the ectopic lesions are unable to synthesize an unknown factor that is postulated to act in a paracrine manner on the stromal cells to induce the expression of 17βHSD2, the enzyme that catalyzes the conversion of E_2 to the less potent estrogen estrone, E_1 (reviewed in Bulun et al., 2006). Thus, the E_2 synthesized via aromatase in the endometriotic lesions is further amplified by the impaired metabolism of E_2 to E_1, due to the lack of progesterone-regulated control over 17βHSD2.

As progesterone resistance is another main feature of the endometriosis phenotype, circulating progesterone has been assessed as a molecular marker of endometriosis. Of the four studies reviewed under the modified QUADAS criteria by May et al., 2010 that assessed serum progesterone as a biomarker of endometriosis, none have revealed any significant alterations between women with and without endometriosis (Fazleabas et al., 1987; Adamyan et al., 1993; Matsuzaki et al., 2006b; Szymanowski, 2007).

Putative biomarkers of endometriosis have been extensively studied and reviewed (May et al., 2010; May et al., 2011; Fassbender et al., 2013; Toor et al., 2014). Unfortunately there is no consensus on the most appropriate biomarkers of disease, partly due to a lack of standardization between studies, small sample sizes, failure to account for menstrual cycle stage, stage of disease, duration of disease, location and lesion type, and varied definitions of the appropriate control group (healthy women, women with pelvic pain but no disease, infertile women, etc.). Our recent systematic review of the fifty-five highest quality papers, scoring six or above on our modified QUADAS criteria, indicated many inconsistencies amongst experimental designs and studies, suggesting that previously reported diagnostic markers might demonstrate clinical utility in larger, more rigorously controlled trials (Toor et al., 2014). Furthermore, because the control groups were so varied the potential utility of several putative biomarkers was likely concealed by confounding factors, and these markers should not necessarily be discounted but rather reassessed (Toor et al., 2014). A concerted effort is being made by clinicians and scientists alike to standardize clinical phenotyping of disease, sample collection, processing, storage, and biobanking to improve

the viability of large-scale, multi-national studies aimed at identifying a useful biomarker of endometriosis (Fassbender et al., 2013; Fassbender et al., 2014; Becker et al., 2014; Rahmioglu et al., 2014b; Toor et al., 2014; Vitonis et al., 2014).

1.6.7: Emerging Areas of Interest

Currently there are several emerging areas of research that show promise in improving biomarker discovery, and assessment in endometriosis research. Proteomics, metabolomics, microRNAs, nerve fibre density, and neurotrophins each offer new and exciting avenues in which to pursue the elusive biomarkers of endometriosis.

1.6.7.1: Proteomics

Studies employing a proteomics approach compare large-scale protein 'fingerprints' between women with and without endometriosis to distinguish between groups. Proteomic studies have been performed on the eutopic endometrium (Fowler et al., 2007; Ten Have et al., 2007; Rai et al., 2010; Stephens et al. 2010; Kyama et al., 2011; Fassbender et al., 2012a; Browne et al., 2012), endometrial fluid aspirates (Ametzazurra et al., 2009), blood samples (Zhang et al., 2006; Liu et al., 2007; Wang et al., 2008; Seeber et al., 2010; Zheng et al., 2011; Fassbender et al., 2012b; Long et al., 2013; Hwang et al., 2014), peritoneal fluid (Ferrero et al., 2007), and urine (Tokushige et al., 2011; El-Kasti et al., 2011; Wang et al., 2014) of women with endometriosis and controls. The mitochondrial proteome has even been compared between women with and without endometriosis (Ding et al., 2010). Three protein peaks identified in this study were capable of distinguishing between women

with and without endometriosis with 87% sensitivity and 86% specificity in a small group of women (Ding et al., 2010). Although each of these studies found proteins that differed between women with endometriosis and controls and reported high sensitivity and specificity within the study, in many cases massive amounts of data were generated, the identity of the proteins were unknown, and the studies could not be replicated due in part to the subjective nature of spot picking after two-dimensional gel electrophoresis, and the poor mass accuracy of mass spectrometry (Van Gorp et al., 2012). Further, when the protein identities were determined by bioinformatics and functional clustering, they generally did not reveal novel proteins or pathways but rather those already known to be associated with endometriosis. For example, Ten Have et al., 2007 reported the dysregulation of one hundred and nineteen proteins in the eutopic endometrium of women with endometriosis compared with controls, and the fifty with the highest fold change were involved in apoptosis, immune reaction, and cell structure. However, a few studies have identified proteins not previously or commonly associated with endometriosis including peroxiredoxin-6, ribonuclease/angiogenin inhibitor 1 (Stephens et al., 2010), cytokeratin 19 (Tokushige et al., 2011), and neurotrophins (Browne et al., 2012), and one study highlighted considerable post-translational modification of proteins as a key factor in disease pathology (Stephens et al. 2010).

While proteomics is not widely employed at present mainly due to its cost, it does offer a new technique to complement others in the search for biomarkers. Additionally, if the goal of the study is not centered around identifying a protein unique to endometriosis, perhaps

our approach to proteomics should be to conduct protein arrays for a limited number of known targets, rather than to isolate a plethora of dysregulated proteins by gel electrophoresis and mass spectrometry, linking them by bioinformatics to pathways already known to be associated with disease. Conversely, if the aim is to screen for novel endometriosis-associated proteins or post-translational modifications, gel electrophoresis followed by mass spectrometry is an option. Although there are small sample sizes in the published reports, concerns of reproducibility, the potential for massive amounts of resultant data, and difficulty identifying and validating of protein hits, many of these concerns are similar to those of other techniques. Therefore proteomics is a promising approach to biomarker mining that has the potential with careful study to yield useful results.

1.6.7.2: Metabolomics

Metabolomics as it relates to endometriosis is only just beginning to be explored. Metabolomics is an emerging area of research that exploits the use of cellular metabolites to create metabolic 'fingerprints' of physiological and pathological states (Nicholson and Lindon, 2008). In theory, a metabolomic profile reflects the molecular events closest to the disease phenotype, as the concentrations of specific metabolites represents the end products of gene expression and provides an overall integrative indication of tissue function within the context of the organism (Nielsen and Oliver, 2005). The use of metabolomics to identify changes in metabolite profiles as a result of endometriosis has been performed in four studies.

The first study focused on comparing lipid profiles between women with and without endometriosis (Melo et al., 2010). The authors identified dyslipidemia (elevated low-density lipoprotein, non-high density lipoprotein cholesterol, triglycerides, total cholesterol, and a lower high-density lipoprotein to total cholesterol profile) in a group of 40 women with surgically confirmed endometriosis compared with 80 healthy controls (Melo et al., 2010). Although their objective was not specifically to assess the suitability of the plasma lipid profile as a biomarker of endometriosis, Melo et al., 2010 provided evidence to support the association of oxidative stress and inflammation in the pathophysiology of endometriosis. A subsequent study by another group demonstrated the ability of eight lipid metabolites in the plasma to act as biomarkers of ovarian endometriosis, supporting the results of the initial study by Melo et al., 2010 (Vouk et al., 2012). After adjusting for age and body mass index (BMI), a model containing phosphatidylcholines and sphingomyelins was able to predict ovarian endometriosis with a sensitivity of 90% and specificity of 84% in a group of 40 surgically confirmed cases, and 52 surgically confirmed controls (Vouk et al., 2012).

The metabolomic profile of serum from women with surgically confirmed disease was compared to surgically confirmed controls in an attempt to identify serum biomarkers specific for Stage I and II endometriosis (Dutta et al., 2012). The study reported a panel of 13 metabolite biomarkers that could be used to detect Stage I and II disease with 82% sensitivity and 91% specificity (Dutta et al., 2012). The final study, performed by the same

group identified increased glucose metabolism, citrate, and succinate in the serum of women with endometriosis compared with controls, and further confirmed results of the preceding studies by demonstrating alterations in reactive oxygen species (Jana et al., 2013).

Even though metabolomics is a relatively new area, it may be possible to find a unique set of metabolites to use as a biomarker of endometriosis. Thus, in addition to the other 'omics' disciplines, metabolomics offers another emerging research avenue to be explored.

1.6.7.3: MicroRNA

MicroRNAs (miRNAs) are short, non-coding RNAs that negatively regulate messenger RNA (mRNA) translation by repressing the protein translational machinery or degrading their target transcripts. A miRNA inserts into the miRNA-induced silencing complex (miRISC) and represses translation by degrading its target mRNA or inhibiting the translational machinery. Target mRNAs contain a short sequence complementary to the 5' end of the miRNA (seed sequence) in their 3'untranslated region (UTR) (Krol et al., 2010). Although only first described a decade ago, over 2000 mature human miRNA sequences have been reported, and are suspected to control approximately 50% of all protein coding genes (Krol et al., 2010). Importantly, the expression of miRNAs, especially in the uterus, is regulated by ovarian hormones (Castellano et al., 2009; Klinge, 2009; Nothnick and Healy, 2010; Nothnick et al., 2010; Kuokkanen et al., 2010; Lessey, 2010).

MiRNAs have been shown to regulate many physiological and pathological conditions. Not surprisingly, several studies have shown aberrations in miRNA expression associated with endometriosis (Pan and Chegini, 2008; Burney et al., 2009; Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011; Ramon et al., 2011; Lin et al., 2012; Braza-Boils et al., 2013; Jia et al., 2013; Laudanski et al., 2013; Suryawanshi et al., 2013; Wang et al., 2013b; Hsu et al., 2014; Braza-Boils et al., 2014; Saare et al., 2014). Although each study was conducted independently, often employing different types of tissue or fluids to quantify miRNAs, hundreds of aberrations linked to endometriosis have been identified, and some redundancy of dysregulated miRNAs exists between studies. However, what is lacking is a link between circulating miRNAs and the mechanism leading to their dysregulation, as many of the miRNAs shown to be elevated in the circulation of women with endometriosis are not shown to be over-expressed in endometriotic lesions (Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011; Ramon et al., 2011; Saare et al., 2014). Nonetheless, circulating miRNAs offer another avenue for non-invasive endometriosis biomarker discovery.

1.6.7.4: Nerve Fibre Density

Over the last ten years there has been increased interest in the presence and increased density of nerve fibres in the eutopic endometrium of women with endometriosis (Tokushige et al., 2006a; Tokushige et al., 2007; Al-Jefout et al., 2007; Al-Jefout et al., 2009; Bokor et al., 2009; Aghaey Meibody et al., 2011; Elgafor El Sharkwy, 2013). While the majority of the other putative diagnostic markers do not relate to the primary clinical

complaint of pelvic pain in women with endometriosis, nerve fibres offer the potential link between symptoms and disease.

While the existence of nerve fibres in the ectopic lesions has been reported, their presence may depend on the type of lesion. Nerve fibres are commonly found in endometriotic adhesions (Tulandi et al., 1998), deep infiltrating disease (Kelm Junior et al., 2008; Wang et al., 2009a; Wang et al., 2009b), and peritoneal implants (Wang et al., 2011), but may be more rare in endometriomas (Al-Fozan et al., 2004; Tokushige et al., 2010; Zhang et al., 2010; McKinnon et al., 2012). While an early study did not find an association between nerve fibres and peritoneal lesions (Tulandi et al., 2001), another study revealed a relationship between the expression of transforming growth factor β1 (TGF- β1) in the nerve fibres, lesion type, and the severity of dysmenorrhea where red lesions and deep infiltrating disease correlated with increased TGF- β1 and patient reported pain (Tamburro et al. 2003). Further, evidence of a direct contact between sensory nerve fibres and peritoneal implants was demonstrated by two independent studies, there was evidence that the lesions had neurotrophic properties (Tokushige et al., 2006b; Mechsner et al., 2007), and that the presence of nerve fibres correlated with the severity of pelvic pain (Mechsner et al., 2009; McKinnon et al., 2012). Thus, the ectopic lesions have increased innervation that may lead to disease-associated pain.

However, the ectopic tissues are not easily accessible and thus the presence of nerve fibres in these tissues will not be an ideal biomarker of endometriosis. The first study to associate

the presence of nerve fibres in the eutopic endometrium found a higher density of small nerve fibres in the functional layer of the endometrium in women with endometriosis as compared to surgically confirmed controls (Tokushige et al., 2006a). The difference in nerve fibres was striking. Small unmyelinated nerve fibres were identified by immunohistochemistry in all 35 women diagnosed with endometriosis, but not in any of the 82 controls (Tokushige et al., 2006a). Results of a subsequent study were similar, and the nerve fibres were classified as sensory and adrenergic fibres (Tokushige et al., 2007). The same group performed a follow-up pilot study to assess the efficacy of employing the detection of nerve fibres in endometrial biopsies to diagnose endometriosis, and reported a sensitivity and specificity of 100% (Al-Jefout et al., 2007). To date, several groups have now demonstrated the use of neural markers in endometrial biopsies as a semi-invasive test to accurately diagnose endometriosis with high sensitivity and specificity (Tokushige et al., 2006a; Tokushige et al., 2007; Al-Jefout et al., 2007; Al-Jefout et al., 2009; Bokor et al., 2009; Aghaey Meibody et al., 2011; Elgafor El Sharkwy, 2013). Only one group has suggested that that the association between nerve fibres and endometriosis may not be specific to endometriosis, but rather indicative of pain symptoms (Zhang et al., 2009). The authors demonstrated the presence of nerve fibres in the endometrium of women with endometriosis, adenomyosis, and fibroids, but not in women without pelvic pain (Zhang et al., 2009). Nevertheless, a recent systematic review of endometrial biomarkers of endometriosis reported that six of the nine highest quality studies (scored 8-9 on the modified QUADAS criteria) identified putative markers relating to nerve fibre growth and cell cycle control (May et al., 2011).

1.6.7.5: Neurotrophins

The neurotrophins are potent neuronal growth factors and mediators of neurogenesis. They are a family of small molecular weight glycoproteins predominantly expressed within the central and peripheral nervous system, and are classically known to promote the development, growth, function, and survival of neurons (reviewed in Chao 2003). The neurotrophin family comprises four ligands: brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951), neurotrophin-3 (NTF3) (Maisonpierre et al., 1990), and neurotrophin-4/5 (NTF5) (Hallbook et al., 1991; Ip et al., 1992) and their respective receptors (reviewed in Chao 2003). The high affinity neurotrophin receptors belong to the the neurotrophic tyrosine kinase receptor (NTRK) family, while the low affinity receptor, p75 neurotrophin receptor (NGFR), belongs to the tumor necrosis factor receptor family (reviewed in Chao 2003). The three NTRK neurotrophin receptors, each exhibit ligand promiscuity. NTRK1 binds NGF with high affinity and NTF3 with a lower affinity; NTRK2 interacts with BDNF with a high affinity, NTF5 with medium affinity, and also NTF3 at a lower affinity; and NTRK3 binds NTF3 with a high affinity (Soppet et al., 1991; Klein et al., 1991; reviewed in Chao 2003; reviewed in Minichiello, 2009). Unlike the NTRK family, NGFR binds all four neurotrophins with a comparable affinity and is considered the low affinity receptor of each neurotrophin (reviewed in Chao 2003; reviewed in Minichiello, 2009). In addition to the NTRK family and NGFR receptor there is an emerging, yet lesser known, neurotrophin co-receptor, sortilin (SORT1). SORT1 has recently been shown to interact with pro-

neurotrophins in the brain and to control their release in either a constitutive or activity-dependent manner (reviewed in Nykjaer and Willnow 2012). It may also be involved in a complex intracellular trafficking network directing proteins to various fates: cell surface expression, secretion, endocytosis, or transport within the cell (reviewed in Nykjaer and Willnow 2012).

In addition to their role in promoting nerve growth and maintenance, activation of the neurotrophin pathways, particularly the BDNF-NTRK2 pathway can induce many different physiological processes which are likely important in both healthy tissues and disease. Specifically the BDNF-NTRK2 interaction induces angiogenesis *in vivo* during vascular remodeling post-Leishmania infection (Dalton et al., 2015) and in a matrigel assay (Kermani et al., 2005), and *in vitro* in endothelial and cancer cells (Kim et al., 2004; Nakamura et al., 2006; reviewed in Kermani and Hempstead, 2007; Blais et al., 2013; Kilian et al., 2014; Lin et al., 2014b; Usui et al., 2014;), cellular proliferation *in vitro* in primary neural progenitors, fibroblasts, macrophages, and cancer cell lines (Glass et al., 1991; Represa et al., 1993; Elkabes et al., 1996; Tervonen et al., 2006; Kawamura et al., 2010; Lawn et al., 2015), adhesion in mouse fibroblast and rat intestinal epithelial cell lines *in vitro* (Zhou et al., 1997; Geiger and Peeper, 2007), and resistance to apoptosis *in vitro* in intestinal epithelial cells, embryonic stem cells, cancer cells, and primary neurons (Douma et al., 2004; Wang et al., 2005; Geiger and Peeper, 2007; Kawamura et al., 2010; Nikoletopoulou et al., 2010; Li et al., 2011). However, the role of BDNF and its receptors in the uterus is not known, and the mechanisms that regulate their uterine expression are

similarly unknown. As each of the aforementioned pathways have been implicated in the pathophysiology of endometriosis, a better understanding of neurotrophins in the uterus is warranted.

Recent evidence has suggested an important role for the neurotrophins in reproductive physiology including participation in endometrial stem cell neurogenesis (Shoae-Hassani et al., 2011), placental development and function (Kawamura et al., 2009; Casciaro et al., 2009; Kawamura et al., 2010; Kawamura et al., 2011; Non et al., 2012), and embryonic development (Kawamura et al., 2005; Kawamura et al., 2007; Kawamura et al., 2012). More importantly, the overexpression of neurotrophins has been linked to reproductive pathologies including premature ovarian failure (Dorfman et al., 2014), endometrial cancer (Bao et al., 2013), and endometriosis (Figure 5) (Anger et al., 2007; Borghese et al., 2010; Browne et al., 2012; Zhang et al., 2012; Barcena de Arellano et al., 2013). Indeed, elevated BDNF and NTF5 expression in the eutopic endometrium (Browne et al., 2012) and elevated NGF and NTF3 are reported in the peritoneal fluid (Barcena de Arellano et al., 2011a; Barcena de Arellano et al., 2013) of women with endometriosis. Finally, the results of a preliminary study suggested that women with endometriosis have elevated circulating BDNF concentrations compared to healthy controls, which decreased after surgical removal of lesions (Giannini et al., 2010).

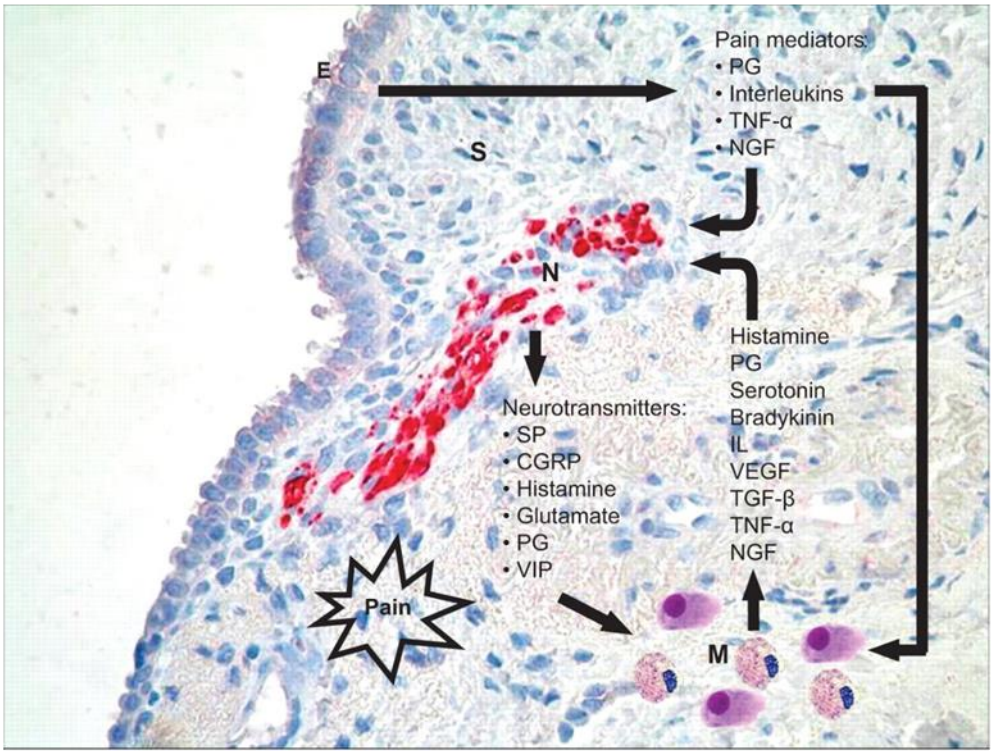

Figure 5: Interaction between Inflammation, Neurotransmitters and Pain in Ectopic Endometriotic Lesions. Inflammation in the ectopic lesions results in the release of pro-inflammatory factors by macrophages and mast cells (M). The endometriotic epithelial (E) and stromal cells (S) express pain mediators including factors that further drive inflammation, and neuronal growth factors including a member of the neurotrophin family, nerve growth factor (NGF). As nerve fibres (N) are often associated with endometriotic lesions, it seems likely that neuronal growth factors including the neurotrophins are in part responsible for initiating growth and maintaining fibre survival, as that is their role in the brain and nervous system. Once nerve fibres have established, they are likely to synthesize neurotransmitters, perhaps contributing to recruitment of additional immune cells or more importantly contributing to disease-associated pain. CGRP: calcitonin gene-related peptide, IL: interleukin, PG: prostaglandin, SP: substance P, TGF-β: transforming growth factor β, TNF-α: tumor necrosis factor α, VEGF: vascular endothelial growth factor, VIP: vasoactive intestinal peptide. Figure reprinted from Barcena de Arellano et al., 2011b, with permission from SAGE Publications.

1.7: Rationale

Endometriosis is a condition that is under-diagnosed as its symptoms mimic other gynecological and gastrointestinal disorders, and clinical biomarkers do not exist. Currently, diagnosis only occurs after laparoscopic visualization of endometriotic lesions, and subsequent histological confirmation of disease. In most cases, the average length of time between a patient presenting with symptoms of disease until confirmed diagnosis is 11.7 years (Ballard et al., 2006). This is problematic because the disease generally worsens over time, and its chronic nature is a burden on the healthcare system with the annual cost approximating $69.4 billion in the U.S. (Simoens et al., 2012; reviewed in Burney and Giudice, 2012) and $1.8 billion in Canada in 2009 (Levy et al., 2011). This is significantly more than comparable chronic conditions (Simoens et al., 2007). As such, the identification of clinical markers of endometriosis was identified as a top research priority by a panel of endometriosis experts (Rogers et al., 2009).

Recently, a putative link between BDNF and women with endometriosis has been established. Preliminary data indicated that women with endometriosis had elevated circulating BDNF as compared to healthy asymptomatic women, which decreased after surgical removal of lesions (Giannini et al., 2010), and that both BDNF and its high affinity receptor, NTRK2, were overexpressed in the uterus of women with endometriosis (Anger et al., 2007; Browne et al., 2012). Even though the presence of nerve fibres in endometrial biopsies appears to be a promising biomarker of endometriosis, in terms of sensitivity, specificity, and replicability, it will forever remain a semi-invasive diagnostic

test. As the neurotrophins are highly expressed in both the peritoneal fluid (Barcena de Arellano et al., 2011a; Barcena de Arellano et al., 2013) and eutopic endometrium of women with endometriosis (Browne et al., 2012), and are readily accessible and quantifiable in the blood, we propose they might provide a safe, fairly non-invasive clinical test for endometriosis.

1.8: Hypothesis

The neurotrophins are potent activators of nerve growth, but are also capable of inducing angiogenesis, proliferation, adhesion, and resistance to apoptosis; pathways implicated in the pathophysiology of endometriosis. Despite this, the expression of neurotrophins in the uterus and the mechanisms regulating their expression are poorly defined. Therefore, the purpose of this thesis was to investigate the expression and regulation of BDNF and its receptors in the mammalian uterus, and to assess circulating BDNF as a biomarker of endometriosis. The overall hypothesis for the studies contained herein is that BDNF is an estrogen-regulated growth factor expressed by the cells of the endometrium that will provide a novel, relatively non-invasive clinical marker of endometriosis in women.

1.9: Objectives

Each of the three studies included in this thesis had a specific hypothesis, and they were conducted in a sequential manner. Collectively the first two studies lay the foundation for future research on uterine neurotrophins, by documenting the presence of neurotrophins in

the mammalian uterus, and identifying a regulatory mechanism of neurotrophin expression in the uterus. The third study demonstrated elevated BDNF concentrations in the peripheral circulation of women with endometriosis versus a control group consisting of symptomatic and asymptomatic women. This study will inspire clinicians and other research groups to assess BDNF as a putative clinical marker of endometriosis in larger patient populations.

1.9.1: Objective 1

The first objective of this Ph.D. thesis was to demonstrate the conserved uterine expression of BDNF and NTRK2 in several mammalian species. The existing literature surrounding BDNF and NTRK2 was narrowly focused on their expression and function within the nervous system. While there were a few reports documenting the presence of BDNF and NTRK2 in non-neuronal tissues, their expression in the uterus was equivocal as some studies identified the uterine expression of the ligand, but not receptor and vice versa, while others failed to localize both ligand and receptor. Therefore, the first objective was to fill a gap in the literature by describing BDNF and NTRK2 expression in the uterus of six mammalian species. As there were sparse reports of BDNF and NTRK2 expression in the uterus, I hypothesized that both BDNF and its high affinity receptor, NTRK2, would be expressed in the mammalian uterus.

1.9.2: Objective 2

The second objective of this Ph.D. thesis was to determine if the ovarian hormones (estrogen and progesterone) participate in the uterine regulation of BDNF, and its receptors

(NTRK2, NGFR, and SORT1) in mice. The results from the first study suggested that the expression of BDNF and NTRK2 might relate to estrous cycle stage, which may explain in part those previous studies that failed to identify BDNF or NTRK2 in the uterus. Data from studies on neurotrophin expression in the brain and nervous system supported their regulation by both estrogen and progesterone. Therefore, I hypothesized that estrogen and progesterone were responsible for the regulation of Bdnf and its receptors in the murine uterus, both during the natural estrous cycle, and in ovariectomized mice exposed to estrogen and progesterone.

1.9.3: Objective 3

Taken together, the results from the first two studies demonstrated that BDNF and each of its receptors are expressed in the endometrium, and that the overexpression of uterine BDNF and its low affinity receptor (NGFR) is supported by estradiol. Thus, BDNF is probably not only susceptible to upregulation by estradiol in the eutopic endometrium, but upregulated in the ectopic endometrium where excess estrogen prevails. Therefore, the final objective of this Ph.D. thesis was to quantify BDNF and other putative biomarkers of endometriosis including NGF, NT4/5, CA-125 and CRP in the plasma of women with and without endometriosis and assess their suitability as clinical markers of disease. I hypothesized that BDNF would provide a novel clinical marker for this enigmatic disease, and would be significantly elevated in women with endometriosis as compared to those without.

Chapter 2

The Brain-Uterus Connection: Brain Derived Neurotrophic Factor (BDNF) and Its Receptor (Ntrk2) Are Conserved in the Mammalian Uterus

Publication:

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2.1 Chapter Introduction

Although mainly recognized for their roles in the central and peripheral nervous system, BDNF and NTRK2 have been described in non-neuronal cells and tissues. In humans and mice, BDNF expression has been observed in platelets (Yamamoto and Gurney, 1990), eosinophils (Noga et al., 2003), dendritic cells (Noga et al., 2008), T cells, B cells, monocytes (Kerschensteiner et al., 1999; Rost et al., 2005), endothelial (Nakahashi et al., 2000), and epithelial cells (Lommatzsch et al., 1999; Hahn et al., 2006). Its expression in reproductive tissues including the rat uterus (Krizsan-Agbas et al., 2003), mouse placenta and amniotic fluid (Kawamura et al., 2009) has been reported. The tissue localization of the high affinity receptor for BDNF, NTRK2, has been more thoroughly assessed

(Shibayama and Koizumi, 1996). NTRK2 was immunolocalized mainly in glandular cells, bone marrow hematopoietic cells, and the epidermis (Shibayama and Koizumi, 1996). More recently, it has been described in reproductive tissues including the ovary (Anderson et al., 2002; Harel et al., 2006), and the endometrium (Anger et al., 2007).

While there are scant reports of BDNF and NTRK2 expression in reproductive tissues, a comprehensive, cross-species analysis showing the presence of both ligand and receptor in the mammalian uterus was lacking. Therefore, the objective of the first paper was to fill a gap in the literature by demonstrating the presence of BDNF and NTRK2 transcripts and protein in the uterus of six mammalian species.

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The Brain-Uterus Connection: Brain Derived Neurotrophic Factor (BDNF) and Its Receptor (Ntrk2) Are Conserved in the Mammalian Uterus

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2.2 Abstract

The neurotrophins are neuropeptides that are potent regulators of neurite growth and survival. Although mainly studied in the brain and nervous system, recent reports have shown that neurotrophins are expressed in multiple target tissues and cell types throughout the body. Additionally, dysregulation of neurotrophins has been linked to several disease conditions including Alzheimer's, Parkinson's, Huntington's, psychiatric disorders, and cancer. Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family that elicits its actions through the neurotrophic tyrosine receptor kinase type 2 (Ntrk2). Together BDNF and Ntrk2 are capable of activating the adhesion, angiogenesis, apoptosis, and proliferation pathways. These pathways are prominently involved in reproductive physiology, yet a cross-species examination of BDNF and Ntrk2 expression in the mammalian uterus is lacking. Herein we demonstrated the conserved nature of BDNF and Ntrk2 across several mammalian species by mRNA and protein sequence alignment, isolated BDNF and Ntrk2 transcripts in the uterus by Real-Time PCR, localized both proteins to the glandular and luminal epithelium, vascular smooth muscle, and myometrium of the uterus, determined that the major isoforms expressed in the human endometrium were pro-BDNF, and truncated Ntrk2, and finally demonstrated antibody specificity. Our findings suggest that BDNF and Ntrk2 are transcribed, translated, and conserved across mammalian species including human, mouse, rat, pig, horse, and the bat.

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2.3 Introduction

Brain derived neurotrophic factor (BDNF) is one member of the neurotrophin family of secreted growth factors which also comprises nerve growth factor (NGF), neurotrophin-3 (Ntf3), and neurotrophin-4/5 (Ntf5). The neurotrophins are classically known for their participation in the development, growth, function, and survival of neurons in both the central and peripheral nervous system [1]. They induce a myriad of actions by signalling through the neurotrophic tyrosine receptor kinase family (Ntrk1 – formerly TrkA, Ntrk2 – formerly TrkB, Ntrk3 – formerly TrkC, and NGFR – formerly p75NTR). BDNF binds with a high affinity to Ntrk2, which has at least three isoforms, a full length transmembrane receptor, and two truncated receptors. Mainly studied in the nervous system, the interaction between BDNF and the full length Ntrk2 receptor has also been shown to activate adhesion,

angiogenesis, apoptosis, and proliferation pathways via the ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and the phospholipase Cγ1Ca^{2+} pathway [1–3]. In addition to participating in many physiological processes, the neurotrophins have been linked to numerous pathologies (Alzheimer's, Parkinson's, Huntington's, cancer) and psychiatric disorders (bipolar, schizophrenia, depression) [1,4,5].

Although abundant in the nervous system, BDNF and Ntrk2 are expressed in other cell types and tissues, and BDNF mRNA is found in the majority of the human body organs [6]. In humans, mature BDNF is sequestered in platelets [7] and released upon their degranulation. As such, BDNF has access to all tissues and organs. Motile cells including activated T cells, B cells, and monocytes have been shown to express BDNF in vitro [8,9], as have eosinophils [10], dendritic cells [11], and endothelial cells [12]. In mice, the visceral epithelium [13], and airway epithelium are significant sources of BDNF [14]. As for Ntrk2, a comprehensive analysis of Ntrk2 immunoreactivity was assessed and it was found to be expressed mainly in glandular cells of the salivary gland, small intestine, colon, endocrine pancreas, bone marrow hematopoietic cells, monocytes/macrophages of the lymph nodes and spleen, and in the epidermis [15]. Previous studies have shown that neurotrophins in the brain are regulated by neuronal activity (Ca++ influx induced transcription) [16], and steroid hormones [17–20], and that tissue-specific expression is driven by multiple promoters [21].

Although the interaction between the BDNF-Ntrk2 ligand-receptor pair has been shown to activate the adhesion, angiogenesis, apoptosis, and proliferation pathways in other body systems, very few studies have addressed their physiological role in reproduction. While BDNF and Ntrk2 expression has been demonstrated in some reproductive tissues including the ovary [22,23], and placenta [24], their uterine expression under physiological conditions has been questionable. BDNF expression was demonstrated by immunohistochemistry in the mouse [25], and human uterus [26,27] and by *in situ* hybridization in the mouse [13], and rat [18] uterus. While Ntrk2 could not be detected in the mouse [13] and human uterus [15], others have been successful [28,29]. To date only one study has looked for the presence of both ligand and receptor simultaneously, in the murine uterus [13]. Moreover, the uterine expression of BDNF and Ntrk2 has not been examined in species other than the mouse, rat, and human.

Herein we present a comprehensive overview of the conserved nature of BDNF and Ntrk2 expression in the uterus of several mammalian species including human, mice, rats, pigs, horses, and bats.

2.4 Materials and Methods

GenBank Accession Numbers

Human BDNF (KC855559), Mouse BDNF (KC855560), Rat BDNF (KC855561), Pig BDNF (KC855563), Horse BDNF (KC855562), Human Ntrk2 (KC855566), Mouse Ntrk2 (KC855567), Rat Ntrk2 (KC855568), Horse Ntrk2 (KC855569).

Cross-Species mRNA and Protein Sequence Alignment

mRNA and protein sequences were obtained for coding regions of the Ntrk2 and BDNF genes from sequences available on NCBI's Nucleotide (www.ncbi.nlm.nih.gov/nuccore). mRNA was aligned across species using mVISTA (http://genome.lbl.gov/vista/ mvista/submit.shtml), and phylogenetic trees were constructed [21–33]. NCBI's Blastn and Blastp were used to compare nucleotide and protein identities and gaps between species.

Animal and Human Samples

Ethics statement. All animal procedures followed research protocols approved by the Animal Research Ethics Board at McMaster University, the University of Guelph Animal Care Committee, and the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Collection of human endometrial tissue samples was approved by the McMaster University and Hamilton Health Sciences Research Ethics Board (REB #10-326-T) and written informed consent was provided by study participants.

Mice. C57/Bl6 mouse uterine horns (n =31) were collected from non-pregnant females aged 8–12 weeks, post-euthanasia and were promptly placed on ice. One uterine horn was stored at -80°C until required. The other was placed in 10% formalin, processed, and embedded in paraffin wax for immunohistochemistry.

Table 1. GenBank accession numbers and Real-Time PCR melting peak

Species and Gene	GenBank Accession	Melting Peak (°C)
Human *BDNF*	KC855559	82
Mouse *BDNF*	KC855560	82
Rat *BDNF*	KC855561	82
Pig *BDNF*	KC855563	84
Horse *BDNF*	KC855562	84
Human *Ntrk2*	KC855566	79
Mouse *Ntrk2*	KC855567	82
Rat *Ntrk2*	KC855568	83
Horse *Ntrk2*	KC855569	80

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Rats. The uterine horns of non-pregnant female Wistar rats (n =11) were graciously provided by Dr. Alison Holloway. Uterine horns were collected at euthanasia and immediately placed on ice. One uterine horn was stored at -80°C until required. The other was placed in 10% formalin, processed, and embedded in paraffin wax for immunohistochemistry.

Humans. Human uterine samples (n= 8) were collected by the Pathology Department at McMaster University Medical Centre (Hamilton, ON, Canada) from patients undergoing a hysterectomy. Samples were immediately transported to the lab, and bisected with one half being frozen for RNA/protein applications, and the other half placed in 10% formalin, processed, and embedded in paraffin wax for immunohistochemistry.

Pigs. Non-pregnant porcine uterus (n= 3) was provided by Dr. Chandra Tayade. Samples were collected at euthanasia, placed on ice, and one half was frozen at -80°C until required. The other was placed in 4% paraformaldehyde, processed, and embedded in paraffin wax for immunohistochemistry.

Horses. Archived uterine punch biopsies previously obtained from five pregnant mares at gestation day 15 (n= 5) were provided by Dr. Keith Betteridge. RNA from three biopsies was available and two biopsies had been processed for immunohistochemistry. Non-pregnant uterine tissue was not available for study.

Bats. All procedures were carried out in accordance with the Policy on the Care and Use of Animals, approved by the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Collection of the uterine horns of fulvous fruit bats was detailed previously [34]. In brief, the bats were trapped alive on Day 21 (n= 6; the day when menstrual bleeding was observed was designated as Day 1). The uterine horns were collected under anesthesia, fixed in 4% paraformaldehyde solution, dehydrated with graded ethanol solution, and then processed for paraffin embedding.

RNA and Protein Extraction

Total RNA was extracted from all mouse, rat, and human endometrial samples using the RNA/Protein Plus kit (Norgen Biotek, Mississauga, ON, Canada). The protocol was modified slightly from the manufacturer's directions. Briefly, approximately 25 mg of frozen uterus was minced with a scalpel, placed in 300 µl of lysis reagent from the kit, and disrupted on ice using a sonicator (Fisher Scientific, Ottawa, ON, Canada) for roughly 5 seconds. Samples were centrifuged at 4°C at 13000 rpm for 2 minutes. Genomic DNA was removed using a column separator from the RNA/Protein Plus kit, and the remainder of the procedure was performed according to the protocol provided. RNA concentration and quality were assessed by spectrometry (Beckman Coulter, Mississauga, ON, Canada). RNA was extracted from horse and pig endometrium using the RNeasy kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's directions. RNA concentration and purity was measured using the GeneQuant pro RNA/DNA calculator (Biochrom Ltd., Cambridge, UK).

Protein extraction from human endometrium (n= 8) and mouse brain as a positive control was performed

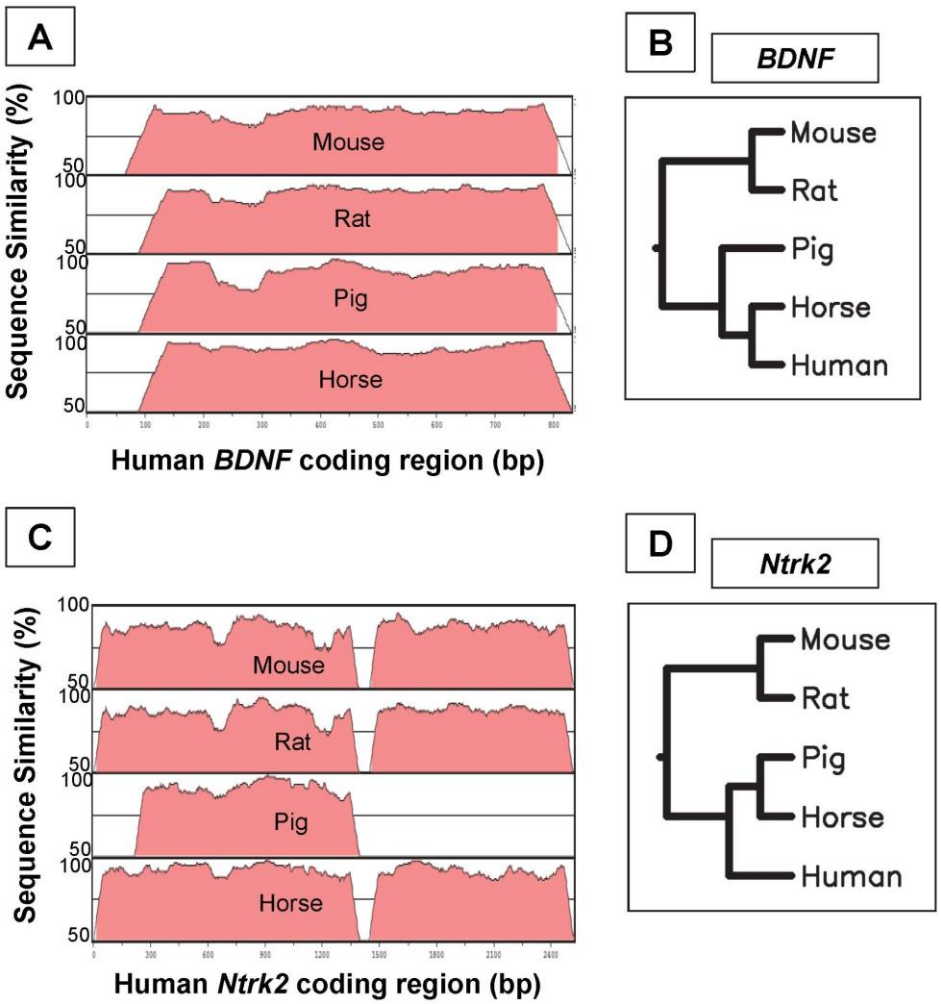

Figure 1. Sequence Homology between Species. Coding regions for BDNF (A) and Ntrk2 (C) were aligned between human, mouse, rat, pig, and horse using mVISTA to show inter-species similarities. Results are displayed as percent conservation between all species as compared to the human sequence. Phylogenetic trees were created for BDNF (B) and Ntrk2 (D) to visually illustrate which species were most closely related. bp: base pairs.

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in 200 µl of RIPA buffer. The tissue was disrupted on ice using a sonicator three times, for 5 seconds. Samples were centrifuged, and the supernatant collected. Protein concentration was measured on a microplate reader at 595 nm using the Bio-Rad protein assay based on the Bradford method (Bio-Rad, Mississauga, ON, Canada).

Real-Time PCR

RNA from mouse, rat, human, pig, and horse was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), according to kit protocol. PCR primers were designed using human GenBank sequences for BDNF mRNA (NM_001143809.1) and Ntrk2 mRNA (NM_006180.3). Primers were designed against a 300 bp span within the coding region of the gene, and whenever possible were designed to span an intron. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used for primer design and primers were tested for hairpins, self-dimers, and hetero-dimers using OligoAnalyzer 3.1

Table 2. Comparison of the coding region of *BDNF* mRNA across species.

Species	Comparison Species	Base Pair Identities (*BDNF* mRNA)	Gaps
Human	Mouse	712/775 (92%)	0%
	Rat	687/750 (92%)	0%
	Horse	690/744 (93%)	0%
	Pig	691/759 (91%)	1%
Mouse	Rat	735/750 (98%)	0%
	Horse	676/750 (90%)	0%
	Pig	681/759 (90%)	1%
Rat	Horse	682/750 (91%)	0%
	Pig	686/759 (90%)	1%
Horse	Pig	692/759 (91%)	1%

doi:10.1371/journal.pone.0094036.t002

(http://www.idtdna.com/analyzer/applications/oligoanalyzer/). Primer sequences for BDNF were (Forward: GAGCTGAGCGTGTGTGACAG, Reverse: CTTATGAA TCGCCAGCCAAT), and for Ntrk2 (Forward: CAATTGTGGTTTGCCATCTG, Reverse: TGCAAAATGCACAGTGAGGT). Primers were ordered from Mobix Laboratory (McMaster University, Hamilton, ON, Canada), and diluted to a working concentration of 10 pmol/µl with DNase/ RNase free water.

cDNA for 3 animals per group was pooled and used to isolate BDNF and Ntrk2 transcripts. Real-Time PCR was performed in triplicate in a 10 µl reaction volume (2 µl pooled cDNA, 5 µl SYBR Green Master Mix (Qiagen), 1 µl forward primer, 1 µl reverse primer, and 1 µl RNase/DNase free water) using the capillary-based LightCycler (Roche Diagnostics, Laval, QC, Canada). The program was denaturation: 95°C for 15 min; amplification: 55 cycles: 95°C for 10 s, 56°C for 5 s, 72°C for 20 s; melting curve: 70–95°C at a rate of 0.1°C per second. Amplification and melt curves were analyzed for each species using the LightCycler software (Roche Diagnostics). PCR products were collected, and sent for sequencing (Laboratory Services, University of Guelph). Each sequence was searched under the BLASTN analysis on the National Center for Biotechnology Information website. Sequences were submitted to NCBI GenBank (accession numbers and PCR product melting temperatures are listed in Table 1).

Assessing Antibody Specificity

Antibody Pre-absorption. Mouse brain sections were cut at a thickness of 4 µm, and incubated with 1) anti-BDNF or antiNtrk2 1:200 (Abcam, Cambridge, MA, USA) (positive control), 2) anti-BDNF or anti-Ntrk2 pre-incubated with an excess of human recombinant protein (BDNF Abcam ab9794 and Ntrk2 Abcam ab56652) at a 5:1 ratio with the antibody, or 3) normal goat serum in lieu of primary antibody. BDNF sections were counterstained with propridium iodide, and visualized using a chicken anti-rabbit Alexa Fluor 488 secondary (Life Technologies, Burlington, ON, Canada). Fluorescence was captured using the Photometrics CoolSnap HQ camera (Roper Scientific, Sarasota, FL, USA) and identical exposure times between positive, preabsorbed, and negative sections. Ntrk2 sections were stained using the ABC kit (Vector Labs, Burlington, ON, Canada) and DAB as a chromogen, and images captured with an Infinity camera (Lumenera Corp., Ottawa, ON, Canada) under 200X magnification on an Olympus IX81 microscope (Olympus, Richmond Hill, ON, Canada).

Human Recombinant Protein Western Blot. Antibody specificity was also assessed by Western Blot (as below) using the same recombinant human BDNF and truncated Ntrk2 proteins as above (Abcam) in a 2X serial dilution.

Immunohistochemistry

Paraffin sections were cut at a thickness of 4 µm for mice (n= 31), rats (n= 11), humans (n= 10), pigs (n= 3), and horses (n= 2). Sections were separately stained for BDNF and Ntrk2 using a 1:200 dilution of rabbit anti-BDNF (Abcam) or rabbit antiNtrk2

Table 3. Comparison of the coding region of BDNF across species.

Species	Comparison Species	Amino Acid Identities (BDNF Protein)	Gaps
Human	Mouse	248/256 (97%)	0%
	Rat	241/249 (97%)	0%
	Horse	240/247 (97%)	0%
	Pig	244/252 (97%)	1%
Mouse	Rat	247/249 (99%)	0%
	Horse	237/249 (95%)	0%
	Pig	241/252 (96%)	1%
Rat	Horse	239/249 (96%)	0%
	Pig	241/252 (96%)	1%
Horse	Pig	240/252 (95%)	1%

doi:10.1371/journal.pone.0094036.t003

Table 4. Comparison of the coding region of *Ntrk2* mRNA across species.

Species	Comparison Species	Base Pair Identities (*Ntrk2* mRNA)	Gaps
Human	Mouse	1211/1397 (87%)	0%
	Rat	2160/2517 (86%)	2%
	Horse	2302/2517 (91%)	2%
	Pig	1090/1180 (92%)	0%
Mouse	Rat	2325/2466 (94%)	0%
	Horse	2155/2471 (87%)	0%
	Pig	1003/1182 (85%)	1%
Rat	Horse	2149/2472 (87%)	0%
	Pig	991/1182 (84%)	1%
Horse	Pig	1105/1184 (93%)	0%

doi:10.1371/journal.pone.0094036.t004

Table 5. Comparison of the coding region of Ntrk2 across species.

Species	Comparison Species	Amino Acid Identities (Ntrk2 Protein)	Gaps
Human	Mouse	772/838 (92%)	2%
	Rat	769/838 (92%)	2%
	Horse	800/838 (95%)	2%
	Pig	376/394 (95%)	0%
Mouse	Rat	809/821 (99%)	0%
	Horse	767/822 (93%)	0%
	Pig	348/393 (89%)	0%
Rat	Horse	768/822 (93%)	0%
	Pig	348/393 (89%)	0%
Horse	Pig	393/452 (87%)	4%

doi:10.1371/journal.pone.0094036.t005

(Abcam), as above. Negative sections were incubated with normal goat serum in lieu of primary antibody. Images were captured by an Infinity camera (Lumenera Corp.) under 200X magnification on an Olympus IX81 microscope. Bat sections (n= 6) were stained at the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, in Beijing, China using anti-BDNF (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and anti-Ntrk2 (Santa Cruz) antibodies as above.

Western Blot

Extracted protein (60 μg) from human endometrium was run on a 4–20% gradient gel (Thermo-Scientific) at 150 V for 50 minutes. Protein was transferred to PVDF membrane (VWR International, Mississauga, ON, Canada) at 40 V for 90 minutes. Blots were blocked for 1 hour at room temperature with 5% skim milk/TBS-T, and subsequently probed with 1:1000 rabbit antiBDNF (Abcam) or 1:1000 rabbit anti-Ntrk2 (Abcam), overnight at 4°C. Anti-Rabbit-ECL secondary (GE, Mississauga, ON, Canada) at a concentration of 1:5000 was applied for 1 hour at room temperature, blots were briefly washed in TBS-T and TBS, then incubated with ECL substrate (Thermo-Scientific) for 5 minutes. Exposures were performed using x-ray film (Thermo-Scientific), and the exposure times were 60, and 45 minutes for BDNF and Ntrk2 respectively.

2.5 Results

Cross-Species mRNA and Protein Sequence Homology

When the coding regions of the BDNF and Ntrk2 genes were compared, they were very homologous between the species examined (human, mouse, rat, pig, horse). The mRNA for both genes had less homology between species as compared to the protein. BDNF mRNA ranged from 90–98% (Table 2), and protein from 95–99% (Table 3). Ntrk2 mRNA ranged from 84– 94% (Table 4), and protein from 87–99% (Table 5). The mRNA coding region from mouse, rat, pig, and horse for both BDNF (Figure 1A) and Ntrk2 (Figure 1C) was aligned against the human sequence and are displayed as percent conservation between all of the aligned species as compared to the human sequence. Phylogenetic trees were created for each mRNA to determine which species were most closely related (Figure 1B, D).

BDNF and *Ntrk2* Transcripts in the Uterus

Primers designed against a 300 bp region of high homology within the BDNF and Ntrk2 coding regions were used to isolate uterine transcripts by Real-Time PCR (Figure 2). Both primer pairs isolated specific products which were verified by sequencing in all species (human, mouse, rat, pig, and horse) except for a nonspecific peak obtained with the Ntrk2 primers in pig uterus. PCR product sequences were submitted to GenBank. Accession numbers are listed in Table 1.

BDNF and Ntrk2 Antibody Specificity

Antibody Pre-absorption. In order to confirm antibody specificity the antibodies used in this study were pre-absorbed using human recombinant proteins and used to stain mouse brain sections by immunohistochemistry. BDNF staining was minimized, and Ntrk2 staining was completely obliterated after antibody pre-absorption as compared to positive control sections (Figure 3A–F), indicating that the antibodies bound to their reported targets. Negative sections were included to show that minimal background staining was observed (Figure 3C,F).

Human Recombinant Protein Western Blot. The human recombinant BDNF and Ntrk2 which were used to pre-absorb the antibodies in 3.3.1 were examined by Western Blot in a 2X dilution. Specific bands of 10, 15, and 20 kDa were observed in the most concentrated dilution of BDNF (0.04 μg) (Figure 3G), and a band of approximately 50 kDa was observed in all dilutions of Ntrk2 (Figure 3H). The recombinant Ntrk2 protein was a truncated version of this receptor, and a band size of 50 was expected.

BDNF and Ntrk2 Expression in the Uterus

Localization of BDNF and Ntrk2 by Immunohistochemistry. The uterine expression of BDNF (Figure 4) and Ntrk2 protein was assessed by immunohistochemistry (Figure 5). In all species examined (human, mouse, rat, pig, horse, and bat) BDNF immunoreactivity was detected in the luminal epithelium, glandular epithelium, myometrium, and vascular smooth muscle, particularly in pig and horse uterus. The uterine expression of Ntrk2 mirrored that of BDNF, being mainly localized in the luminal epithelium, glandular epithelium, and myometrium.

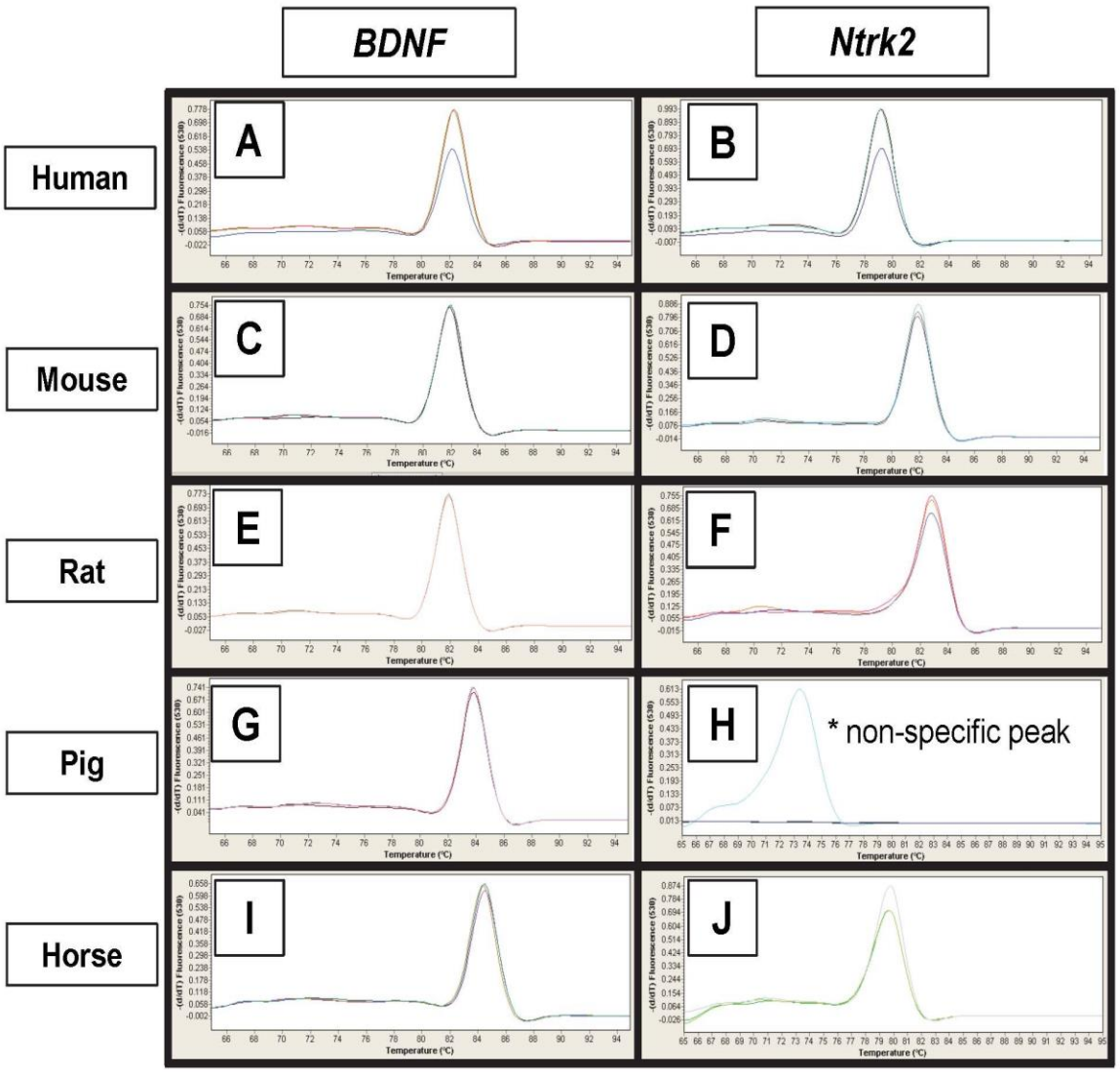

Figure 2. Isolation of Uterine BDNF and Ntrk2 Transcripts. Real-Time PCR melting peaks for uterine BDNF and Ntrk2 in human (A, B), mouse (C, D), rat (E, F), pig (G, H), and horse (I, J). Both primer pairs isolated specific products which were verified by sequencing in all species except for a non-specific peak (*) obtained with the Ntrk2 primers in pig uterus (H). doi:10.1371/journal.pone.0094036.g002

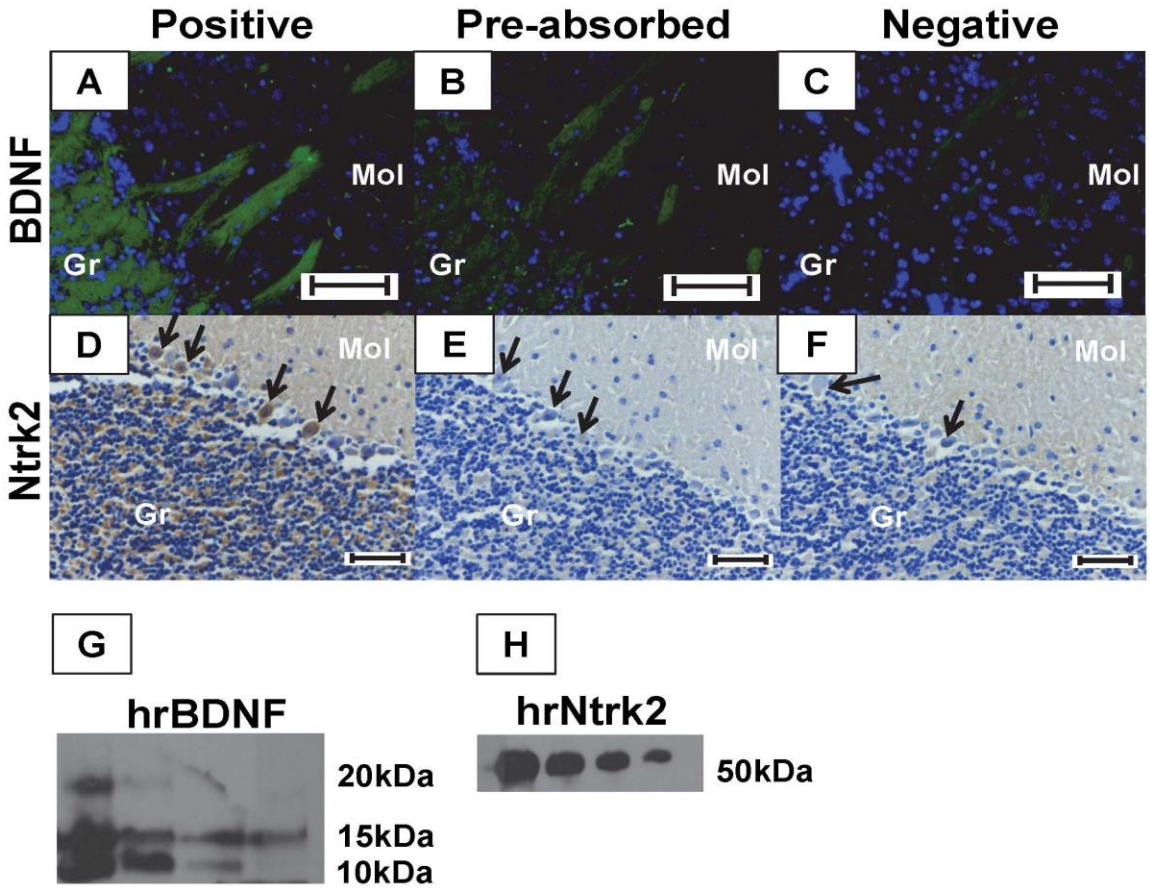

Figure 3. Assessing Antibody Specificity. Mouse brain sections were stained by immunohistochemistry with anti-BDNF (A) or Ntrk2 (D) antibodies as positive controls, or with antibody which had been pre-incubated with human recombinant BDNF (B) or Ntrk2 (E) protein, or with normal goat serum as a negative control (C, F). Decreased or absent staining was observed in pre-incubated sections as compared to positive controls (A vs. B; D vs. E). A 2X serial dilution of human recombinant BDNF (G) and truncated Ntrk2 (H) revealed bands of the appropriate sizes by Western Blot. Green: BDNF, brown: Ntrk2, blue: nucleus. Arrowheads: Purkinje cells, Gr: Granular layer, Mol: Molecular layer. doi:10.1371/journal.pone.0094036.g003

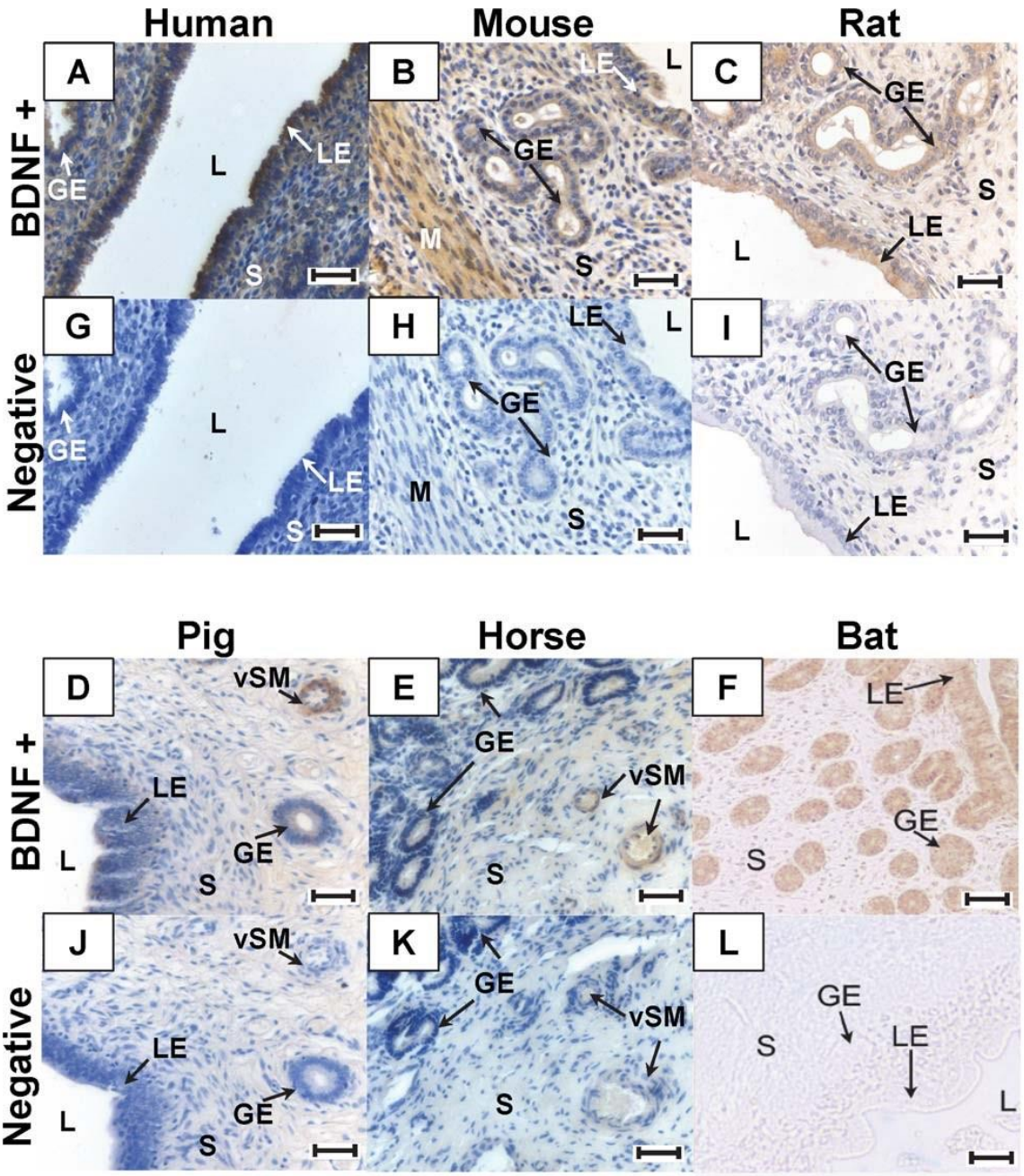

Figure 4. Immunohistochemical localization of BDNF in the Uterus. Uterine sections were stained for BDNF (A–F) using DAB as a chromogen (brown stain) or incubated with normal goat serum as a negative control (G–L). BDNF immunoreactivity was observed in human (A), mouse (B), rat (C), pig (D), horse (E), and bat (F) uterus. It localized to the luminal epithelium (LE), glandular epithelium (GE), smooth muscle of the myometrium (M) and vascular smooth muscle (vSM) in the mammals examined. Original image magnification was 200X. Scale bar represents 50 mm. L: lumen, S: stroma. doi:10.1371/journal.pone.0094036.g004

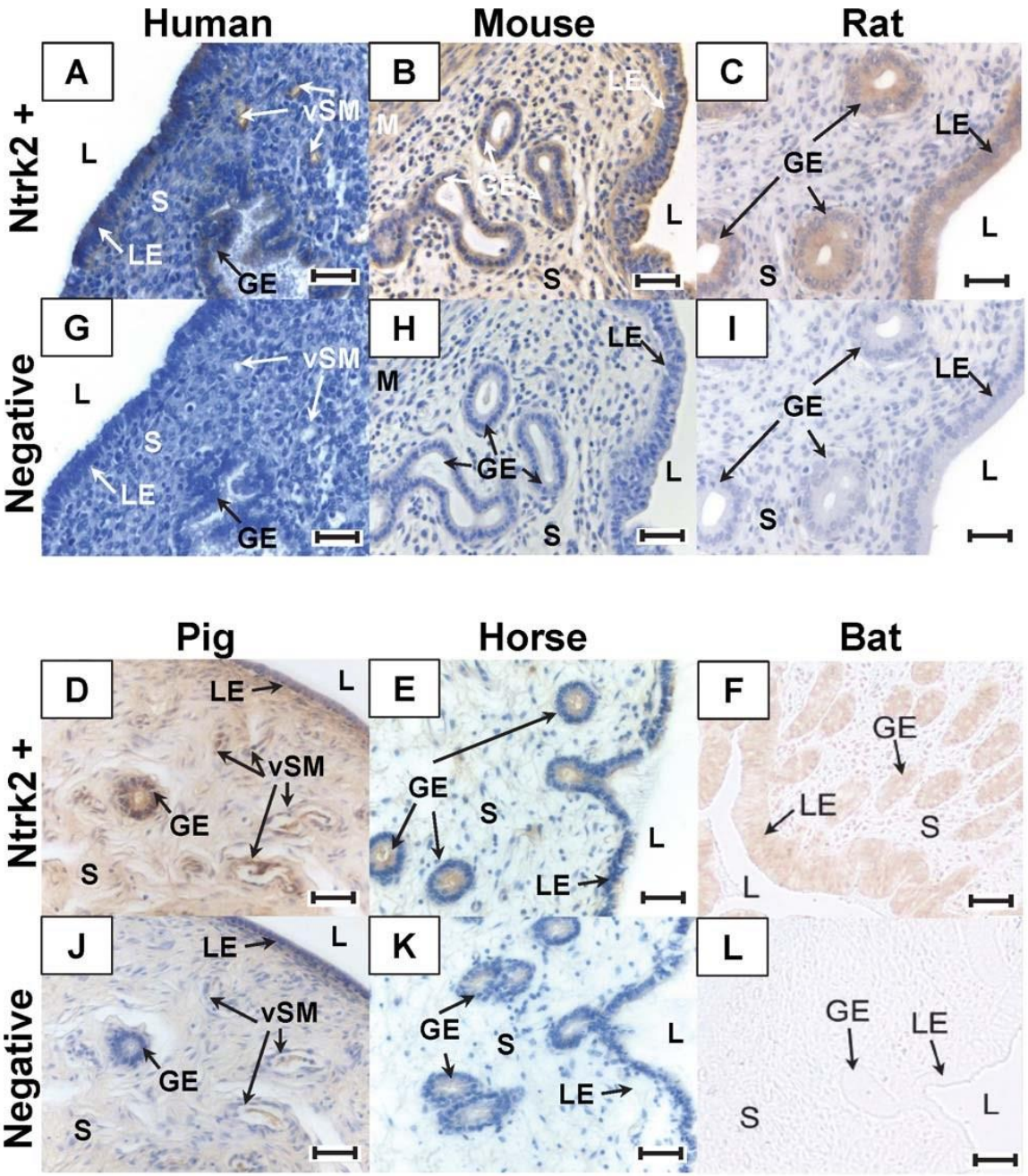

Figure 5. Immunohistochemical localization of Ntrk2 in the Uterus. Uterine sections were stained for Ntrk2 (A–F) using DAB as a chromogen (brown stain) or incubated with normal goat serum as a negative control (G–L). Ntrk2 immunoreactivity was observed in human (A), mouse (B), rat (C), pig (D), horse (E), and bat (F) uterus. It localized to the same areas as its ligand BDNF. Ntrk2 was observed in the luminal epithelium (LE), glandular epithelium (GE), smooth muscle of the myometrium (M) and vascular smooth muscle (vSM) in the mammals examined. Original image magnification was 200X. Scale bar represents 50 mm. L: lumen, S: stroma.

doi:10.1371/journal.pone.0094036.g005

BDNF isolation in the Human Uterus by Western Blot. Human endometrium from hysterectomy patients was probed for BDNF (Figure 6A) expression by Western Blot using mouse brain as a positive control. In all nine women, several bands were observed when the anti-BDNF antibody was used to probe the uterine homogenate. Faint 15 and 20 kDa bands were observed in some patients (Figure 6A) and in the mouse brain (Figure 6A: 9). A 25 kDa band was observed in the mouse brain, but not in the human uterus. A band of approximately 35 kDa was seen in all women, and in the mouse brain. However, in the uterine homogenates a doublet was found as compared to a single band in the mouse brain, and in patient 5. Blots were subsequently stripped and probed for beta-actin as a loading control.

Ntrk2 isolation in the Human Uterus by Western Blot. The same samples of human endometrium and mouse brain were probed for Ntrk2 (Figure 6B) expression by Western Blot. A single or double band of roughly 40 kDa were observed in some women (Figure 6B) and in mouse brain (Figure 6B: 9).

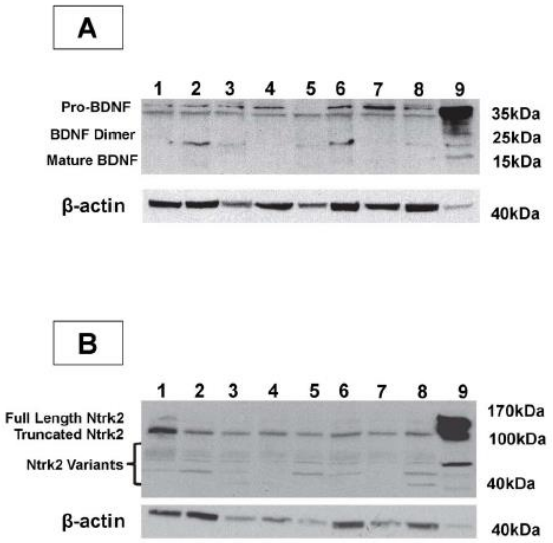

Figure 6. BDNF and Ntrk2 Expression in the Human Uterus. Uterine homogenates were collected from hysterectomy patients and probed for BDNF (A) and Ntrk2 (B) by Western Blot, using mouse brain as a positive control. Uterine samples were loaded in lanes 1–8, and mouse brain homogenate in lane 9. doi:10.1371/journal.pone.0094036.g006

A larger band of approximately 55 kDa, which was much more abundant in the mouse brain, was observed in all endometrial samples as a faint double band. A 100 kDa band which was heavily expressed in the mouse brain was observed in all uterine homogenates. Finally, a larger band of 120 kDa was seen in the positive control, and very faintly in a few of the human uteri. Blots were stripped and probed for beta-actin as a loading control.

2.6 Discussion

Here, using complementary molecular techniques, we demonstrated the conservation of the coding region of BDNF and Ntrk2 across several mammalian species, the mRNA expression of both genes within the uterus, and the uterine localization of both proteins in two species that menstruate (humans and bats [34]), and four that do not (mice, rats, pigs, and horses). Additionally, we have shown that several protein isoforms of each gene were present in the human uterus, and that the antibodies employed in this study were specific to BDNF and Ntrk2 respectively. BDNF and Ntrk2 are part of the complex messenger system that is the neurotrophins, which regulate several physiological pathways, and thus we suggest are potentially important to uterine function.

Our results show that both BDNF and Ntrk2 are highly conserved across the mammalian species studied, with protein sequences having greater homology than mRNA sequences. This was not entirely unexpected, as in some cases multiple codons exist for a single amino acid, and thus a base-pair substitution in the mRNA sequence might not alter the protein. Over time, as each of the species studied evolved, silent mutations in the genes likely arose. During evolution, Gotz et al., suggest that BDNF was more highly conserved than NGF across vertebrates [35]. In our study the PCR primer pairs designed to isolate BDNF and Ntrk2 were capable of doing so in the uterus of all animals (except for Ntrk2 in the porcine uterus), and both antibodies employed in this study demonstrated specific uterine immunoreactivity for BDNF and Ntrk2 in each of the six mammals examined, supporting high sequence homology amongst orthologs over evolution.

Antibody specificity in the current study was ascertained in two ways. Firstly, by ensuring bands of the appropriate size were seen when Western blot

was performed with human recombinant BDNF and Ntrk2. Secondly, mouse brain sections (positive control tissue) were stained for BDNF and Ntrk2 with primary antibodies which had been pre-absorbed with BDNF and Ntrk2, respectively. In sections incubated with pre-absorbed BDNF primary antibodies the staining was less intense than the positive control, which had been stained with anti-BDNF, but more intense than the negative control. Ideally pre-absorption obliterates all staining as the antibody should be completely bound by the excess protein. In the case of pre-absorbed BDNF, some of the BDNF bound to the antiBDNF antibody may have bound to endogenous Ntrk2 receptors, and thus given a faint signal when the secondary antibody was applied. Ntrk2 staining in the mouse brain was obliterated by preabsorption. The results of the antibody specificity tests indicated that the antibodies used for immunohistochemistry and Western blot were specific and capable of detecting BDNF and Ntrk2 within the mammalian uterus.

While there are a few studies demonstrating the independent expression of BDNF and Ntrk2 in the uterus, the results of the present study are the first to show that both ligand and receptor are co-expressed, and co-localized in the uterus of several mammalian species. Our results show BDNF and Ntrk2 expression in the glandular epithelium, luminal epithelium, vascular smooth muscle, and myometrium of the human, mouse, rat, pig, and bat uterus. A similar pattern of expression was also observed in the uterus of the pregnant mare. This is the first comprehensive and cross-species comparison of BDNF and Ntrk2 mRNA, and protein in the mammalian uterus. Even though BDNF expression has been seen in uterine pathologies [36,37], BDNF and Ntrk2 expression in the non-pregnant, healthy uterus has been equivocal. Although there are sparse reports of BDNF in the mouse [13,25], rat [18], and human [26,27] uterus, and Ntrk2 in the human uterus [28,29], others have not been able to localize the Ntrk receptor family in the murine [13] nor human uterus [15]. However, the latter study [15] published in 1996, may not have been able to detect Ntrk2 owing to limitations in the sensitivity of PCR techniques then available. Additionally, the co-localization of BDNF and Ntrk2 demonstrated in this study contrasts the results of Lommatzsch et al. (1999) [13], where BDNF mRNA was only observed in the uterine epithelium and stroma, not myometrium, and Ntrk2 immunoreactivity was not observed at all. Again, this may have been due to methodological limitations. The probe designed for in situ hybridization may not have detected all forms of BDNF (pre-, pro-, etc.), and if that particular form was present in the myometrium it would have falsely appeared negative. Also, Ntrk2 appears to exist in low abundance in the uterus; the exposure length to obtain a positive Western blot band is one hour, after loading 60 μg of protein homogenate. Perhaps the antibody used in the previous report was not as sensitive as the antibody employed in this study.

Neurotrophin signalling and regulation is complicated for several reasons: each receptor can bind more than one ligand with varying affinity, multiple splice and transcript variants of ligands and receptors exist, several post-translational modifications may be present, ligands are first translated as pro-proteins which bind receptors, and ligands can exist as monomers or dimers [1]. Thus, the expression of BDNF and Ntrk2 were demonstrated by Western blot in the human endometrium to gain insight into which isoform predominates. A doublet band of roughly 35 kDa was found to be the most widely expressed form of BDNF in the uterus. These bands are likely pro-BDNF which has previously been reported to have a similar mass [38,39]. Smaller bands of approximately 15 kDa likely represent the mature form of BDNF, and are less abundant than the larger bands. It has been suggested that pro-BDNF and mature BDNF have opposing functions. Specifically, pro-BDNF inhibits nerve growth and BDNF promotes and sustains it [40,41]. As for Ntrk2, variability was seen between patients for the bands lower than 100 kDa, but a band at approximately 100 kDa was consistent amongst them all. This band likely represents a truncated version, of which there are two at 95 kDa, of the 140 kDa receptor [42–46].

We speculate that the abundant BDNF and Ntrk2 isoforms found in the human uterus may serve to inhibit the classical BDNF-Ntrk2 pathways, and also prevent nerve growth into a tissue that is degraded and shed in a cyclical manner. However, the degree to which nerves innervate the endometrial layer of the uterus under physiological and pathological conditions remains under debate [47–50]. In support

of our hypothesis, expression of the truncated Ntrk2 was capable of inhibiting sensory nerve innervation of the mammary gland in response to mature BDNF [51]. BDNF and Ntrk2 have also previously been shown to activate the adhesion [5,52–55], angiogenesis [56,57], apoptosis [5,53,58–60], and proliferation [59,61] pathways, mainly in the brain and nervous system. Each of these pathways is also of paramount importance in the reproductive processes of the female mammal. However, little is known about the role of BDNF and Ntrk2 in reproductive physiology. While the literature supporting BDNF expression, particularly in the brain and serum, during pregnancy is growing [62–65], its specific function is still unclear. One group has reported that paracrine BDNF/Ntrk2 signalling induced cytotrophoblast differentiation, proliferation, and survival in an *in vitro* model [25,30], while another showed that BDNF inhibited neurite outgrowth in a superior cervical ganglion/ myometrium explant co-culture [18]. While the role of BDNF/ Ntrk2 in reproductive physiology remains a mystery we suggest that this signaling pathway is potentially important in normal uterine physiology and pathology.

Herein we have given a complete and comprehensive overview of BDNF and Ntrk2 in the mammalian uterus. Firstly, gene conservation was demonstrated for both BDNF and Ntrk2 across species. Secondly, transcripts for both BDNF and Ntrk2 were isolated in the uterus of several mammals. Thirdly, the antibodies were confirmed to be specific for the proteins of interest. Fourthly, protein translation and localization was demonstrated by immunohistochemistry in menstruating and non-menstruating species, and finally the prominent BDNF and Ntrk2 isoforms were identified in the human endometrium. As several of the major pathways central to reproductive biology have been reported to be induced by BDNF-Ntrk2 binding, we suggest that the function of this ligand-receptor pair within the mammalian uterus merits further attention.

2.7 Acknowledgments

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Author Contributions

Conceived and designed the experiments: JMW NAL WGF. Performed the experiments: JMW WGF. Analyzed the data: JMW LW HW WGF. Contributed reagents/materials/analysis tools: HW WGF. Wrote the paper: JMW NAL HW WGF.

2.8 References

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Chapter 3

Estrogen Induced Changes in Uterine Brain-derived Neurotrophic Factor and Its Receptors

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3.1 Chapter Introduction

BDNF expression in the brain and nervous system is, at least in part, regulated by the ovarian hormones (Solum and Handa, 2002; Kaur et al., 2007; Meyer et al., 2012; reviewed in Pluchino et al., 2013). Interestingly, in the brain the expression of BDNF is also spatially regulated, indicating that hormones may induce BDNF expression in certain tissues or cell types but not others (Solum and Handa, 2002). While the fluctuation of NTRK2, one of the BDNF receptors, in the murine brain over the estrous cycle suggests its regulation by the ovarian hormones (Spencer et al., 2008), gonadectomy failed to affect NTRK2 expression

in the rat hippocampus in another study (Solum and Handa, 2002). Thus, the regulation of NTRK2 in the brain by estrogen and/or progesterone remains equivocal.

Additional evidence supporting the regulation of BDNF by the ovarian hormones outside of the brain and nervous system is the presence of an estrogen response element in the BDNF gene (Sohrabji et al., 1995), the significantly higher plasma concentrations reported in women in the secretory phase of the menstrual cycle versus the proliferative phase (Begliuomini et al., 2007), and the positive correlation between circulating BDNF and circulating E_2 in women (Pluchino et al., 2009).

While both estrogen and progesterone participate in the regulation of BDNF in the brain, the mechanisms regulating BDNF and its receptors (NTRK2, NGFR, and SORT1) in the uterus is entirely unknown. As the uterus is a major target tissue for both estrogen and progesterone, it is imperative to understand their role in the uterine regulation of BDNF and its receptors. Thus, the second objective of this thesis was to determine if the ovarian hormones (estrogen and progesterone) participate in the uterine regulation of BDNF, and its receptors (NTRK2, NGFR, and SORT1) in mice.

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Estrogen induced changes in uterine brain-derived neurotrophic factor and its receptors

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3.2 Abstract

STUDY QUESTION: Are brain-derived neurotrophic factor (BDNF) and its receptors, NTRK2, NGFR and SORT1, regulated by ovarian steroids in the uterus?

SUMMARY ANSWER: BDNF and its low affinity receptor, nerve growth factor receptor (NGFR), are regulated by estradiol in the uterus.

WHAT IS KNOWN ALREADY: Recent studies have revealed a central role for neurotrophins in placental development, endometrial stem cell neurogenesis, endometrial carcinoma and endometriosis. Complex signaling pathways involving BDNF and its receptors are regulated by ovarian hormones in the brain, however their expression and regulation in the uterus is poorly defined.

STUDY DESIGN, SIZE, DURATION: This experimental animal study involved a total of 80 mice.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Female C57BL/6 mice ($n = 50$) were monitored daily for estrous cycle stage, and uterine horns were collected. A second group of mice ($n = 30$) were ovariectomized and given estradiol, progesterone, estradiol + progesterone, or saline for 4 days. Uterine expression of BDNF and its receptors were quantified by real-time PCR and western blot, and localized using immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: During the estrous cycle, expression of BDNF, NTRK2 and SORT1 remained constant, while NGFR declined 11-fold from pro-estrus through to diestrus ($P = 0.005$). In ovariectomized mice, estradiol treatment increased uterine expression of mature BDNF greater than 6-fold ($P = 0.013$, 25 kDa; $P = 0.003$, 27 kDa), pro-BDNF 5-fold ($P = 0.041$, 37 kDa band; $P = 0.046$, 40 kDa band), and NGFR 5-fold ($P < 0.001$) when compared with other treatments. NTRK2 and SORT1 were unaffected by ovarian hormones. NGFR was primarily localized in epithelial cells in mice in diestrus or in ovariectomized mice treated with progesterone ($P \leq 0.001$; $P \leq 0.001$, respectively). In contrast, NGFR switched to a stromal localization in ovariectomized mice administered estradiol ($P = 0.002$).

LIMITATIONS, REASONS FOR CAUTION: This study was performed in one only species.

WIDER IMPLICATIONS OF THE FINDINGS: Results of this study demonstrate the uterine regulation of BDNF and NGFR by estradiol, and highlight the striking difference between hormone exposure during the estrous cycle and daily estradiol exposure after ovariectomy on neurotrophin expression in the uterus. The results also show the spatial regulation of NGFR in the uterus in response to ovarian hormones. Sustained estrogen exposure, as seen in estrogen-dependent disease, may alter the delicate neurotrophin balance and inappropriately activate potent BDNF-NTRK2 pathways which are capable of contributing to endometrial pathology.

STUDY FUNDING/COMPETING INTERESTS: This study was supported by the Canadian Institutes of Health Research (CIHR) (W.G.F.), a NSERC Discovery Grant (W.G.F.), and a Vanier Canada Graduate Scholarship-CIHR (J.M.W.). J.M.W. is a member of the CIHR sponsored Reproduction and Early Development in Health training program. The authors declare no conflicts of interest.

Key words: estrogen / BDNF / NGFR / NTRK2 / sortilin

3.3 Introduction

Although mainly recognized for their supportive function within the nervous system, brain-derived neurotrophic factor (BDNF) and its high affinity receptor neurotrophic tyrosine receptor kinase 2 (NTRK2) have been shown to participate in ovarian development (Dorfman et al., 2011), follicular development (Kerr et al., 2009) and oocyte survival (Dorfman et al., 2014). The neurotrophins are also important in endometrial physiology where they participate in endometrial stem cell neurogenesis (Shoae-Hassani et al., 2011) and normal placental development (Kawamura et al., 2009, 2011; Non et al., 2012). However, the overexpression of neurotrophins is associated with reproductive pathologies including premature ovarian failure (Dorfman et al., 2014), endometrial cancer (Bao et al. 2013) and endometriosis (Borghese et al., 2010; Browne et al., 2012; Barcena de Arellano et al., 2013).

The neurotrophins are small molecular weight proteins that act in the nervous system to promote neuronal development, differentiation, growth and maintenance (reviewed in Chao, 2003). The neurotrophin signaling network is complex. Neurotrophins can be translated as proproteins and cleaved into their active forms (Mowla et al., 2001, Gray and Ellis, 2008) or they can induce signaling cascades in their pro-forms (Lee et al., 2001; Koshimizu et al., 2009). Generally, the two forms have opposing functions (reviewed in Chao and Bothwell, 2002; Teng et al. 2010). The neurotrophin family comprises four ligands, BDNF, nerve growth factor (NGF), neurotrophin 3 (NTF3) and neurotrophin 4 (NTF4), and four receptors: neurotrophic tyrosine receptor kinase (NTRK) 1, NTRK2, NTRK3, and the nerve growth factor receptor (NGFR) (reviewed in Chao, 2003; Reichardt, 2006). Although all four neurotrophins bind to NGFR with similar affinities (Chao, 2003; Reichardt, 2006), and their pro-protein forms have been shown to bind to this receptor as well (Lee et al., 2001), they are more selective in binding the NTRKs. NGF binds to NTRK1, BDNF and NTF4 to NTRK2, and NTF3 to NTRK3, each with high affinity (reviewed in Chao, 2003). Another lesser known neurotrophin co-receptor, sortilin (SORT1), has been shown to interact with pro-neurotrophins in the brain and to control their release (reviewed in Nykjaer and Willnow 2012). SORT1 is also involved in intracellular trafficking, directing proteins to various fates: cell surface expression, secretion, endocytosis or transport within the cell (reviewed in Nykjaer and Willnow, 2012).

The interaction between BDNF and NTRK2 is not only capable of inducing neuronal development, differentiation, growth and maintenance, activation of the BDNF-NTRK2 pathway also induces angiogenesis (Kermani et al. 2005, Nakamura et al. 2006), proliferation (Tervonen et al., 2006; Kawamura et al., 2010), adhesion (Zhou et al., 1997; Douma et al., 2004; Geiger and Peeper, 2007) and resistance to apoptosis (Douma et al. 2004, Wang et al. 2005, Geiger and Peeper, 2007; Kawamura et al., 2010; Li et al., 2011). Each of these pathways is inextricably linked to reproduction, but the mechanisms regulating the uterine expression of neurotrophins remain unknown.

Both estrogen (Singh et al., 1995; Gibbs, 1998, 1999, Jezierski and Sohrabji, 2000, 2001; Berchtold et al., 2001; Liu et al., 2001; Solum and Handa, 2002; Scharfman and Maclusky, 2005; Pan et al., 2010; Tang and Wade, 2012) and progesterone (Kaur et al., 2007; Jodhka et al., 2009; Meyer et al., 2012; Su et al., 2012; Atif et al., 2013) regulate BDNF and its receptors in the brain, and we propose that their uterine regulation occurs in a similar manner. The

aims of this study are to determine whether uterine BDNF, NTRK2, NGFR and SORT1 are affected by: (i) the acute, naturally occurring hormone fluctuations of the estrous cycle, and (ii) daily exposure to the ovarian hormones in ovariectomized mice. Here, we contrast the relatively stable expression of BDNF and its receptors over the estrous cycle with the significant up-regulation of uterine BDNF and its low affinity receptor NGFR in response to prolonged exposure to estradiol. Additionally, we document for the first time the presence of NGFR and SORT1 in the uterus.

3.4 Materials and Methods

Ethical approval

All procedures were approved by the animal research ethics board, McMaster University, Hamilton, ON, Canada (AUP 12-04-13).

Mice

Sexually mature female C57BL/6 mice (n = 80) were purchased at 8 weeks of age from Charles River, and housed in a specific pathogen-free facility with a 12 h light/dark cycle, standard rodent chow, and water *ad libitum*.

Cycling mice

Mice (n = 50) were randomly selected for estrous cycle monitoring. Animals were acclimated to vaginal lavage using sterile saline and a curved eyedropper for a 2-week period. Lavage was dried on a glass slide, and stained with a rapid Giemsa (Sigma-Aldrich, Oakville, ON, Canada) protocol. Briefly, slides were fixed in methanol for 5 min, air dried, and stained with Giemsa for 5 min. Estrous cycle stage was assessed on a daily basis by vaginal cytology (Wood et al., 2007; Caligioni, 2009; Byers et al., 2012). Animals were euthanized at each stage of the estrous cycle (pro-estrus n = 8; estrus n = 18; metestrus n = 9; diestrus n = 15) by anesthetic overdose (isoflurane, Pharmaceutical Partners of Canada, Inc., Richmond Hill, ON, Canada). Uterine horns were immediately removed and stored at -80°C.

Ovariectomy and hormone replacement

In the second experiment, sexually mature female mice (n = 30) were ovariectomized, and allowed to recover for 2 weeks. Mice were randomly assigned to treatment groups as outlined in Fig. 1, using previously established methods and doses (Domino and Hurd, 2004; Gillgrass et al., 2005; Salgado et al., 2009, 2011). All groups except the OVX group were primed for 3 days with 5 µg of 17-β estradiol (EMD Millipore, Billerica, MA, USA) by subcutaneous injection. After 2 days of rest, animals were given 5 µg of estradiol, 500 µg of progesterone, 5 µg of estradiol plus 500 µg of progesterone (EMD Millipore), or saline by subcutaneous injection for 4 days. Animals were euthanized, and uterine horns were collected as described above.

RNA and protein extraction

RNA and protein were extracted simultaneously from one uterine horn using the RNA/Protein Purification Plus kit (Norgen Biotek Corp., Thorold, ON, Canada). Approximately 30 mg of uterine horn was cut and sonicated in 300 µl lysis buffer on ice for 30 s, three times. RNA was extracted following the manufacturer's protocol and quantified by Nanodrop (Thermo Scientific, Wilmington, DE, USA). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad).

Real-time PCR

Real-time PCR primers (Table I) were designed against the coding region of genes (to capture all isoforms) using sequences from NCBI Nucleotide, and Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were purchased from IDT Technologies (Coralville, IA, USA). PCR product was sequenced (Laboratory Services, University of Guelph, Guelph, ON, Canada), and BLASTed to

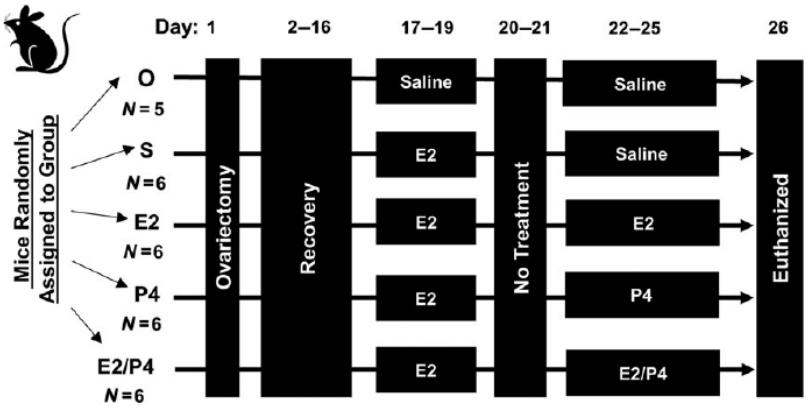


Figure 1 Experimental design for the ovariectomy and hormone replaced mice. Female mice ($n = 30$) were ovariectomized, and allowed to recover for 2 weeks. Mice were randomly assigned to one of five treatment groups (O, S, E2, P4, or E2/P4), and received daily subcutaneous injections of 5 μg of 17-β estradiol (E2), 500 μg of progesterone (P4), 5 μg of estradiol plus 500 μg of progesterone (E2/P4), or saline (S) as a vehicle according to the regimen and timing outlined. O: ovariectomized control group (no hormone exposure).

Table I Real-time PCR primers and information.

	Primers	Anneal (°C)	Melt Peak (°C)	Cycles	Accession number
BDNF	F: GCCCAACGAAGAAAACCATA R: TCAGTTGGCCTTTGGATACC	56	87	55	KF982302
NTRK2	F: CGAGGTTGGAACCTAACAGC R: TTACCCGTCAGGATCAGGTC	62	82	60	KF982303
NGFR	F: GAAGCTGCTCAATGGTGACA R: CACAGAGATGCTCGGTTCTG	58	90	55	KF982304
SORT1	F: TATGCCCCGAATTCCTAGTG R: CCACCTCACATGCAATGTTC	56	87	55	KF982305
GAPDH	F: TGTTCCTACCCCCAATGTGT R: ATGTAGGCCATGAGGTCCAC	56	85	55	KF982306

confirm its identity. Sequences were submitted to NCBI's GenBank and are listed in Table I. Plate-based real-time PCR was performed in duplicate (95°C 5 min, denaturation: 95°C 10 s; annealing: see Table I 20 s; elongation: 72°C 15 s; melting curve: 65–97 2.5°C/s) using the Roche LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) and the SYBR Green I Master Mix (Roche). Relative quantification was performed with *Gapdh* as a reference gene using the Roche LightCycler software, which calculates an efficiency corrected normalized ratio of target gene to *Gapdh* using a mathematical algorithm developed by Roche. Bar graphs represent the group mean plus standard error of measurement (SEM).

For real-time PCR, *Gapdh* was used as a reference gene. Before relative quantification, a one-way ANOVA was used to determine if significant differences existed in crossing points between groups. No significant differences in *Gapdh* were observed between estrous cycle phases ($P = 0.179$) nor between groups of the mice receiving hormone supplementation ($P = 0.271$, data not shown).

Western blot

Total uterine protein (20 μg) was run on a 4–20% gradient gel (Thermo Scientific, Burlington, ON, Canada) under reducing conditions at 150 V for 50 min, and transferred to PVDF (VWR International,

Table II Western blot information.

	Primary antibody concentration	Source	Exposure length (minutes)
BDNF	1:1000	Abcam (ab62011)	60
NTRK2	1:200	Abcam (ab18987)	60
NGFR	1:2000	Abcam (ab8874)	2
SORT1	1:2000	Abcam (ab16640)	3
β-tubulin	1:5000	Abcam (ab6046)	1

Mississauga, ON, Canada) at 40 V for 90 min. Skim milk/TBS-T (5%) was used to block for 1 h at 1:5000; then blots were incubated with enhanced chemiluminescence (ECL) substrate (Thermo-Scientific) for 5 min. X-ray film (Thermo-Scientific) was used for imaging; exposure times are listed in Table II. Blots were stripped using Restore Western Blot stripping buffer (Thermo-Scientific), and rinsed in TBS prior to incubation with another primary antibody. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-Tubulin was employed as the reference gene for the western blots. No differences in β-tubulin were observed in cycling mice ($P = 0.086$) nor in ovariectomized mice receiving hormone supplementations ($P = 0.327$, data not shown).

Immunohistochemistry

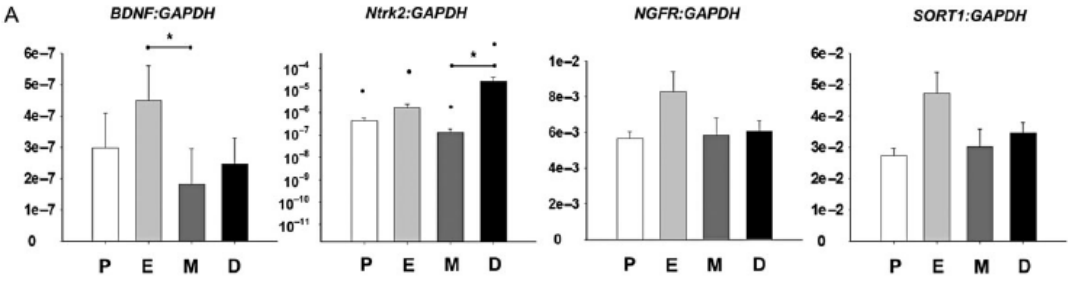
One uterine horn was fixed in 10% formaldehyde, processed, and embedded in paraffin. Uterine cross sections were cut at 4 µm, deparaffinized, and stained for BDNF (ab9794, Abcam, Cambridge, MA, USA, 1:200), NTRK2 (ab56652, Abcam, 1:200), NGFR (ab8874, Abcam, 1:100), and SORT1 (ab16640, Abcam, 1:500) using 1% BSA in PBS as a diluent. In lieu of primary antibody, negative sections were incubated with the blocking solution in the Rabbit Vectastain ABC kit (Vector Labs, Burlington, ON, Canada). The ABC kit was used as per manufacturer's protocol, and DAB was employed as a chromogen (including negative sections). Images were captured with an Infinity camera (Lumenera Corp., Ottawa, ON, Canada) and Olympus IX81 microscope (Olympus, Richmond Hill, ON, Canada).

Quantification of NGFR staining

Four random images of uterine cross sections per mouse were obtained from mice in all cycle phases and treatment groups ($n = 3$ per phase or group). Luminal epithelial, glandular epithelial and stromal cells were counted (100 cells per type) and the percent staining positive for NGFR was calculated.

Data and statistical analysis

Within our data, there were values non-detectable by real-time PCR or western blot. There are several methods to handle non-detectable data points including: assigning these data a value of 0, the limit of detection for the assay, the square root of the limit of detection, or a random number between the limit of detection and zero (Newton and Rudel, 2007; Fievet and Della Vedova, 2010; Ballenberger et al., 2012; Boyer et al., 2013). We assigned a random number between the limit of detection for the gene or protein of interest and zero using the random number generator in the SigmaStat software package (SigmaStat 3.5 Systat Software, Inc., Chicago, IL, USA) because this method will randomly skew the data toward or away from zero, rather than always skewing it in the same direction. Statistical outliers in the data were identified by Grubb's test (http://graphpad.com/quickcalcs/Grubbs1.cfm) for $N > 6$, and the Dixon's Q test for a single outlier, for smaller sample sizes (http://contchart.com/outliers.aspx). Outliers were removed prior to analysis. Any other sample omissions were due to technical error. The number of non-detects, outliers, and omissions are in Supplementary Fig. S1. Real-time PCR and western blot data were compared by one-way ANOVA (SigmaStat 3.5 Systat Software, Inc., Chicago, IL, USA) and Tukey post *hoc* test. Data that were not normally distributed were analyzed by ANOVA on rank's and Dunn's post *hoc* test performed. A *P*-value of <0.05 was considered significant. Bars on the graphs represent the mean plus the standard error of measurement (SEM). Uterine localization of NGFR was compared by t-test.



B

Densitometry Values (% β-tubulin)		Proestrus	Estrus	Metestrus	Diestrus	P value
BDNF	25 kDa	70.5 ± 24.0	39.2 ± 26.6	49.1 ± 11.6	31.2 ± 7.43	0.425
	27 kDa	28.9 ± 9.44	71.9 ± 22.0	54.0 ± 19.1	31.3 ± 9.77	0.263
	37 kDa	39.0 ± 14.0	20.7 ± 11.5	34.6 ± 10.5	10.7 ± 2.07	0.137
	40 kDa	143.2 ± 44.5	95.2 ± 34.9	122.2 ± 16.9	35.7 ± 7.33	0.107
NTRK2	55 kDa	74.1 ± 10.3	67.7 ± 12.3	89.0 ± 22.4	65.5 ± 12.3	0.691
NGFR	75 kDa	111.1 ± 15.3^{A}	103.2 ± 24.8^{A}	63.8 ± 27.5^{A,B}	6.61 ± 2.20^{B}	***0.005***
SORT1	100 kDa	126.8 ± 36.1	91.4 ± 24.3	172.3 ± 69.5	147.1 ± 16.1	0.130

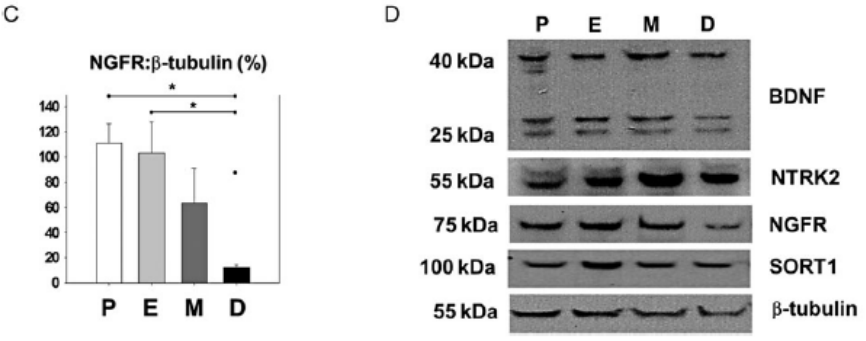


Figure 2 BDNF and its receptors in the cycling mouse uterus. Quantification of *Bdnf* ($n = 8, 17, 8, 14$), *Ntrk2* ($n = 7, 17, 8, 14$), *Ngfr* ($n = 8, 18, 9, 15$), and *Sort1* ($n = 8, 18, 9, 15$) transcripts using *Gapdh* as a reference gene (**A**). Densitometry values for BDNF ($n = 8, 11, 9, 10$), NTRK2 ($n = 7, 12, 9, 10$), NGFR ($n = 7, 12, 8, 9$) and SORT1 ($n = 8, 12, 9, 10$), expressed as a % loading control using β-tubulin (**B**). Graph of the statistically significant differences in NGFR expression over the estrous cycle (**C**). Representative western blot images showing immunoreactive bands for BDNF, NTRK2, NGFR, SORT1 and β-tubulin which was used as the reference gene for densitometry (**D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by an asterisk (*) above the graph, or by different superscripts in the table (B). Outliers were not included in statistical analysis, but are denoted by a dot on the graph if they fell within its range. E: estrus, D: diestrus, M: metestrus, P: pro-estrus.

3.5 Results

BDNF expression in the cycling mouse uterus

Bdnf transcripts were decreased (P = 0.031) in metestrus compared with estrus (Fig. 2A). When BDNF expression was assessed by western blot, four bands (~25, 27, 37, and 40 kDa) were observed in 37 of 39 uteri (Fig. 2B and D). No differences in the 25, 27, 37 or 40 kDa BDNF bands (P = 0.425, 0.263, 0.137, 0.107 respectively; Fig. 2B and D) were observed over the estrous cycle.

BDNF receptor expression in the cycling mouse uterus

Ntrk2 transcripts were elevated in diestrus compared with metestrus (P = 0.017; Fig. 2A). NTRK2 55 kDa protein (Fig. 2B and D), an isoform we previously demonstrated in the human uterus and mouse brain using another NTRK2 antibody (Wessels et al., 2014), remained stable over the estrous cycle (P = 0.691). The long (140 kDa) and truncated (95 kDa) forms of NTRK2 were below the limit of detection, even after an hour exposure. *Ngfr* transcripts were unaltered across the estrous cycle (P = 0.221; Fig. 2A). However NGFR protein decreased over the estrous cycle with levels at diestrus being significantly lower (P = 0.005) than those at pro-estrus or estrus (Fig. 2B–D). Transcripts and protein for SORT1 were unaffected by estrous cycle stage (P = 0.104, P = 0.130; Fig. 2A, B and D).

Uterine localization of BDNF and its receptors in the cycling mouse uterus

BDNF and NTRK2 were co-localized in the luminal epithelium, glandular epithelium, stroma and smooth muscle in the cycling mouse uterus (representative images, Fig. 3). NGFR was also present in all uterine cell types (Figs 3 and 4), but its expression in the luminal epithelium was dependent on whether there was a dominance of estrogen (pro-estrus, estrus, metestrus) or progesterone (diestrus) (Fig. 4A and B). NGFR expression increased (P < 0.001) in the luminal epithelium at diestrus when compared with other cycle stages (Fig. 4A). NGFR expression was absent in the internal layer of smooth muscle in the myometrium, but present in the external layer (Fig. 3). SORT1 remained consistently expressed in the luminal and glandular epithelium (Fig. 3).

Hormonal regulation of BDNF in the mouse uterus

Estrogen and progesterone increased *Bdnf* transcripts above ovariectomized controls, estrogen treated, and progesterone treated alone (P = 0.002; Fig. 5A). Treatment with estradiol significantly increased all quantified isoforms of BDNF in the mouse uterus (Fig. 5B–D). The 25 kDa band increased 6-fold above estrogen primed mice given saline (P = 0.013), and the 27 kDa band increased >7-fold (P = 0.003) above those given saline or progesterone. Estrogen treatment also significantly increased (P = 0.041) the 37 kDa form of BDNF above mice receiving saline. Additionally, estrogen treatment enhanced the 40 kDa band (P = 0.046) when compared with those treated with progesterone only.

Hormonal regulation of BDNF receptors in the mouse uterus

No significant change in uterine *Ntrk2* transcripts was identified (P = 0.066, Fig. 5A). The 55 kDa band was not changed by hormonal treatment (P = 0.788; Fig. 5B and D). The full-length (140 kDa) and truncated (95 kDa) NTRK2 receptors were not quantifiable by western blot, after a 1 h exposure. No differences in *Ngfr* transcripts in the uterus were observed in the ovariectomized mice supplemented with exogenous hormones (P = 0.131; Fig. 5A). NGFR expression in the uterus was significantly increased (P < 0.001) by estradiol treatment when compared with saline and P4 treated animals (Fig. 5B–D). Estrogen and progesterone co-treatment increased *Sort1* transcripts in the uterus above mice treated with estrogen alone, or saline (P = 0.007; Fig. 5A). This difference in SORT1 was not observed at the protein level (P = 0.503; Fig. 5B and D).

Uterine localization of BDNF and its receptors in the hormone replacement mouse uterus

BDNF and NTRK2 were located in the luminal epithelium, glandular epithelium, stroma and smooth muscle in the mouse uterus of all treatment groups (representative images from mice treated with estradiol in Fig. 6). Mice treated with estradiol

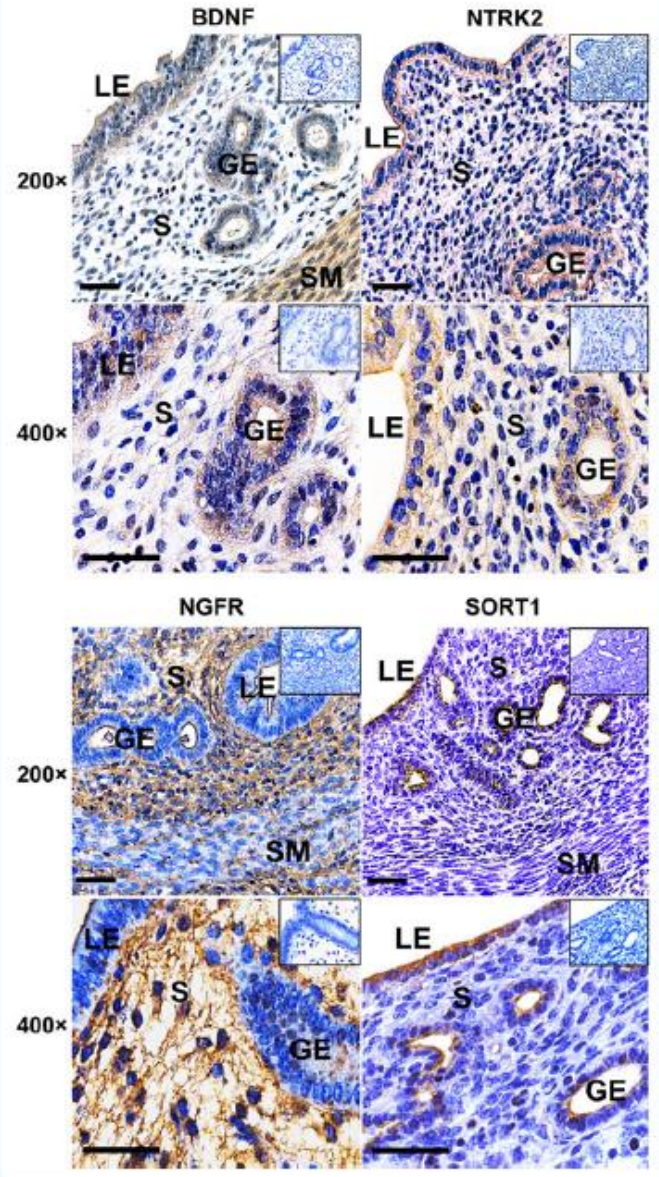


Figure 3 BDNF and receptor localization in the cycling mouse uterus. BDNF, and NTRK2 were co-localized in the luminal epithelium, glandular epithelium, stroma, and smooth muscle in the cycling mouse uterus. Expression of NGFR was also present in all uterine cell types but its localization was dependent on whether there was a dominance of estrogen (pro-estrus) or progesterone (diestrus) (see Fig. 4). SORT1 remained consistently expressed in the luminal and glandular epithelium. Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200, ×400. Scale bar = 50 μm. $n = 8$ (pro-estrus), 18 (estrus), 9 (metestrus), 15 (diestrus). GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle. E: estrus, D: diestrus, M: metestrus, P: pro-estrus.

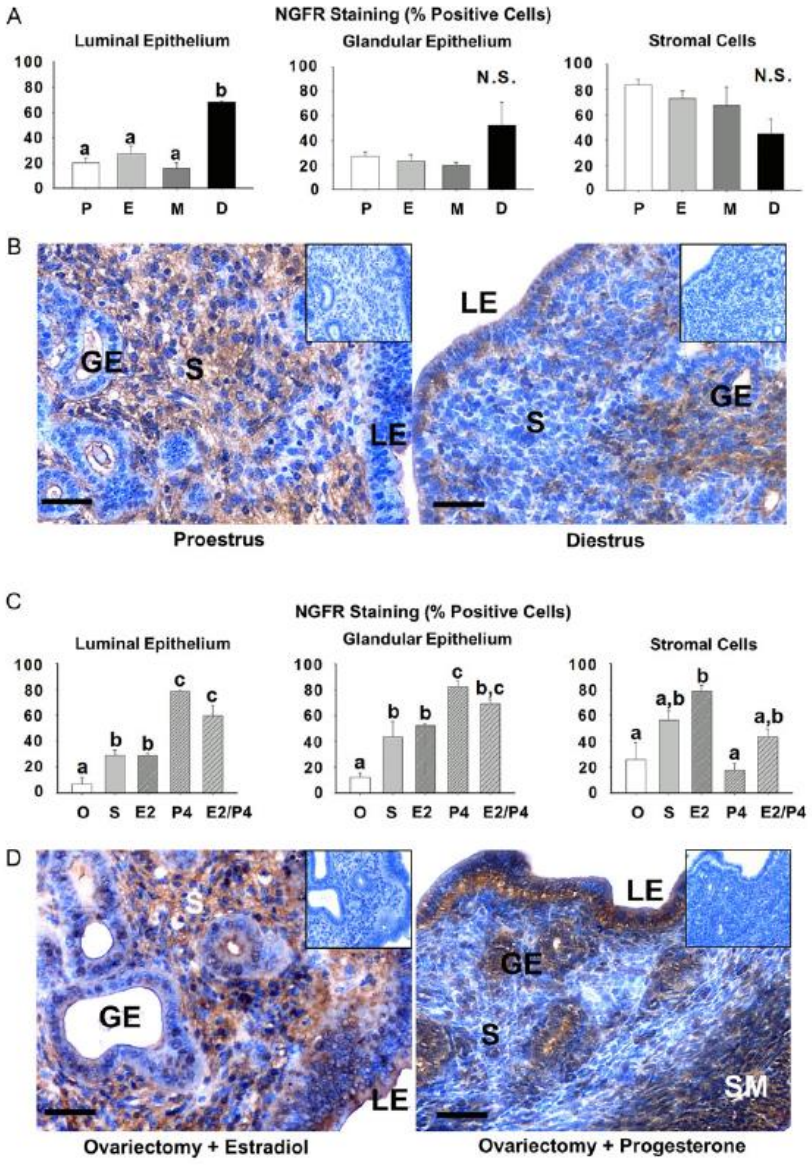


Figure 4 NGFR localization in response to estrogen versus progesterone. In cycling mice, NGFR expression was increased in the luminal epithelium at diestrus ($P < 0.001$) compared with the other cycle phases (**A**, **B**). In ovariectomized mice, the administration of estradiol increased NGFR expression in the stromal cells ($P < 0.001$) when compared with animals given progesterone. The pattern of expression switched to the luminal ($P < 0.001$) and glandular epithelium ($P = 0.002$) when mice were given progesterone (**C**, **D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by different superscripts above the bars. Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200. Scale bar = 50 μm. $n = 3$ per group. E: estrus, D: diestrus, M: metestrus, P: pro-estrus. GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle. O, S, E2, P4, E2/P4: treatment groups according to Fig. 1. N.S.: not statistically significant.

had enhanced BDNF expression, particularly in stromal cells. NGFR was found in all uterine cell types (Figs 4 and 6) but, as in cycling mice, its localization was dependent on whether mice were exposed to estrogen or progesterone (representative images from mice treated with estradiol in Fig. 6). NGFR expression was increased in the stromal cells of ovariectomized mice given estrogen ($P = 0.002$) when compared with mice given progesterone or the ovariectomized controls (group O), and its expression switched to the luminal ($P < 0.001$) and glandular epithelium ($P \leq 0.001$) in mice given progesterone (Fig. 4C). SORT1 was located on the apical side of the glandular epithelium, and occasionally, the luminal epithelium (representative images from mice treated with progesterone in Fig. 6).

3.6 Discussion

Emerging evidence suggests an important role for BDNF in uterine physiology and pathology. Herein we show that BDNF and its low affinity receptor NGFR are regulated by estradiol in the uterus. We contrast the expression of uterine BDNF and its receptors during the 4-day estrous cycle with expression in response to daily estradiol exposure during hormone replacement, as summarized in Fig. 7.

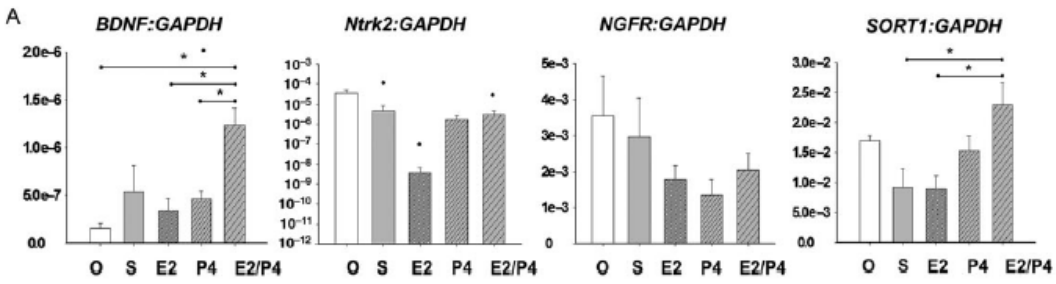
Estrogen regulates BDNF expression in the uterus

In ovariectomized mice, daily estrogen significantly increased all of the BDNF isoforms quantified. BDNF can be a monomer (13 kDa), dimer (26 kDa), or pro- (42 kDa) protein, and can undergo post-translational modifications (Mowla et al., 2001; Teng et al., 2005; Pruunsild et al., 2007; Matsumoto et al., 2008; Koshimizu et al., 2009). Stability studies suggest BDNF dimers are stable, even under reducing blot conditions (Radziejewski et al., 1992; Kolbeck et al., 1994; Pan et al., 1998). Thus, the 25, 27, 37 and 40 kDa bands are likely dimerized and pro-BDNF, with and without post-translational modification. Although progesterone affects BDNF expression in the brain (Kaur et al., 2007; Jodhka et al., 2009; Meyer et al., 2012; Su et al., 2012; Atif et al., 2013) and nervous system (Gonzalez et al., 2004, 2005; De Nicola et al., 2006; Gonzalez Deniselle et al., 2007; Cekic et al., 2012), and BDNF is expressed in luteinized granulosa cells (Dominguez et al., 2011), progesterone did not alter uterine BDNF. Our results concur with Coughlan et al. (2009) where progesterone did not alter BDNF expression in response to neuronal injury. As Jodhka et al. (2009) reported that progesterone was capable of increasing BDNF in the brain but medroxyprogesterone was not, we speculate that the form of progesterone employed affects induction of BDNF.

While this is the first report of estrogen-induced BDNF expression in the uterus, previous studies in the brain support a role for estrogen in BDNF regulation (Toran-Allerand et al., 1992; Miranda et al., 1993; Singh et al., 1995; Sohrabji et al., 1995; Gibbs, 1998, 1999; Jezierski and Sohrabji, 2000, 2001; Berchtold et al., 2001; Liu et al., 2001; Solum and Handa, 2002; Scharfman and Maclusky, 2005; Pan et al., 2010; Tang and Wade, 2012). Additionally, circulating levels of BDNF strongly correlate with estradiol (Pluchino et al., 2009), and fluctuate over the menstrual cycle in women (Begliuomini et al., 2007), and BDNF can be induced by estrogen in the rat uterus (Krizsan-Agbas et al., 2003). Here we have shown that daily estrogen exposure after ovariectomy significantly increases uterine BDNF, but the hormonal fluctuations of the murine estrous cycle do not.

Estrogen regulates BDNF receptors in the uterus

The uterine expression of NGFR decreased over the estrous cycle, and increased in response to estrogen supplementation, while no hormonal regulation of NTRK2 or SORT1 was observed. We postulate that estrogen stabilizes NGFR or increases



B

Densitometry Values (% β-tubulin)		OVX	Saline	Estradiol	Progesterone	Estradiol + Progesterone	P value
	E2 primed	–	+	+	+	+	N/A
BDNF	25 kDa	$43.7 \pm 14.5^{A,B}$	8.48 ± 3.41^{B}	53.4 ± 13.1^{A}	$16.1 \pm 4.61^{A,B}$	$33.3 \pm 8.14^{A,B}$	***0.013***
	27 kDa	$29.9 \pm 11.8^{A,B}$	5.87 ± 2.40^{A}	73.4 ± 18.5^{B}	10.5 ± 2.46^{A}	$46.0 \pm 13.7^{A,B}$	***0.003***
	37 kDa	$24.7 \pm 9.36^{A,B}$	10.9 ± 4.00^{A}	53.4 ± 12.3^{B}	12.3 ± 2.42^{A}	21.5 ± 4.52^{A}	***0.041***
	40 kDa	$44.8 \pm 23.5^{A,B}$	$29.8 \pm 11.9^{A,B}$	231.7 ± 67.3^{A}	21.7 ± 4.22^{B}	$47.7 \pm 9.52^{A,B}$	***0.046***
NTRK2	55 kDa	51.6 ± 23.1	64.0 ± 17.8	86.0 ± 15.0	28.8 ± 11.7	80.5 ± 6.36	0.788
NGFR	75 kDa	$61.4 \pm 42.4^{A,B}$	9.85 ± 3.71^{A}	265.0 ± 34.5^{B}	5.39 ± 2.48^{A}	$44.0 \pm 8.29^{A,B}$	***<0.001***
SORT1	100 kDa	216.3 ± 28.0	172.4 ± 37.9	134.6 ± 35.4	154.7 ± 31.7	174.3 ± 19.4	0.503

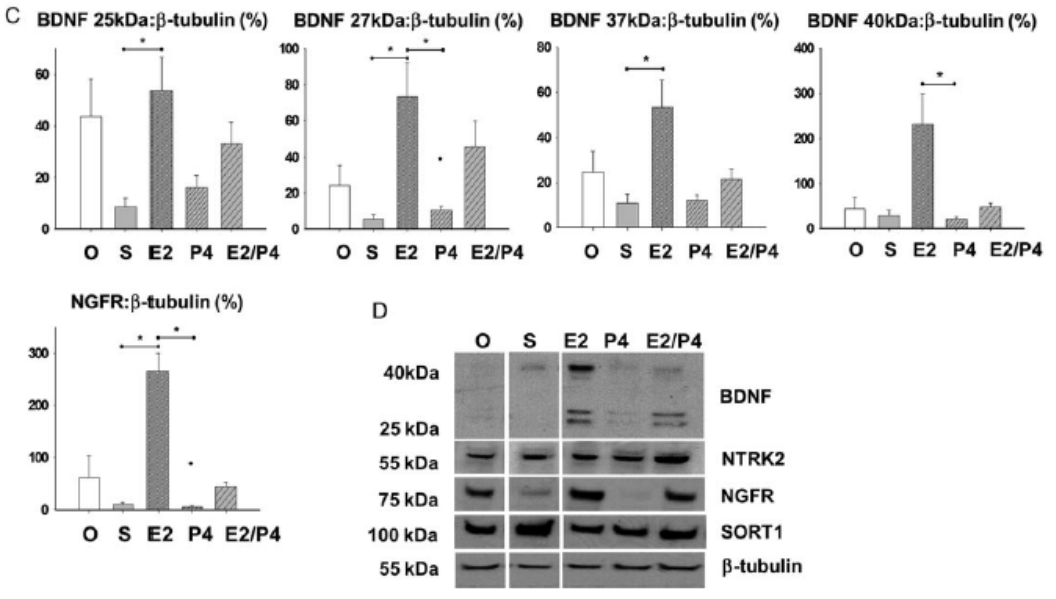


Figure 5 Hormonal regulation of BDNF and its receptors in the mouse uterus. Ovariectomized mice were assigned to treatment groups as outlined in Fig. 1. Quantification of *Bdnf* ($n = 5, 6, 6, 5, 6$), *Ntrk2* ($n = 5, 5, 5, 6, 5$), *Ngfr* ($n = 5, 6, 6, 6, 6$) and *Sort1* ($n = 4, 6, 6, 6, 6$) transcripts using *Gapdh* as a reference gene (**A**). Densitometry values for BDNF ($n = 5, 6, 6, 6, 6$), NTRK2 ($n = 5, 5, 6, 6, 6$), NGFR ($n = 4, 6, 6, 5, 6$) and SORT1 ($n = 5, 6, 6, 6, 6$), expressed as a % loading control using β-tubulin (**B**). Graph of the statistically significant differences in BDNF and NGFR expression in response to exogenous hormones (**C**). Representative western blot images showing immunoreactive bands for BDNF, NTRK2, NGFR, SORT1 and β-tubulin which was used as the reference gene for densitometry. Each gene quantified by densitometry was run on the same membrane; the images have been cropped to reflect the experimental groupings in the graphs (**D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by an asterisk (*) above the graph, or by different superscripts in the table (B). Outliers were not included in statistical analysis, but are denoted by a dot on the graph if they fell within its range. O, S, E2, P4, E2/P4: treatment groups according to Fig. 1. OVX: ovariectomy.

its half-life, as *Ngfr* transcripts are not affected by estradiol. Alternately, estrogen may enhance translation of transcripts, without increasing their quantity (signal amplification). The precise mechanism of estradiol action is unclear, but is likely via indirect regulation of the NGFR protein. Further, NGFR was spatially regulated in the uterus; expression shifted from stromal to epithelial cells when ovariectomized animals were given estrogen versus progesterone.

Regulation of BDNF receptors by estradiol and progesterone in the brain, nervous system (Gibbs and Pfaff, 1992; Sohrabji et al., 1994a,b; Jezierski and Sohrabji, 2001; Brito et al., 2004; Hasan et al., 2005; De Nicola et al., 2006; Spencer et al., 2008; Anesetti et al., 2009; Pan et al., 2010; Cekic et al., 2012; Tang and Wade, 2012) and ovary (Lara et al., 2000) have been reported. Interestingly, in Hasan et al. (2005), acute estrogen exposure in sympathetic neurons did not affect NGFR expression, but chronic exposure did. Here we have shown that uterine NGFR expression decreases over the estrous cycle, and increases in response to daily estrogen exposure after ovariectomy, while other BDNF receptors remain stable. We have also demonstrated the spatial regulation of NGFR in response to ovarian hormones.

BDNF and receptor expression in ovary intact cycling mice when compared with ovariectomized and estradiol replaced mice

In mice, the estrous cycle likely occurs too quickly to significantly affect uterine neurotrophins. Although transcripts for *Bdnf* and *Ntrk2* varied over the estrous cycle, BDNF, NTRK2, and SORT1 expression remained stable and NGFR declined from pro-estrus through diestrus. This decline would increase the local bioavailability of BDNF and signaling through the BDNF-NTRK2 pathways in the uterus during the latter part of the cycle. Thus, under physiological conditions the neurotrophic milieu of the uterus is controlled by NGFR. However, when mice were exposed to daily high dose estrogen, which models the chronic estrogen present in endometriotic lesions in women with endometriosis (Noble et al., 1996; Huhtinen et al., 2012) or other estrogen-dependent diseases, the exposure had profoundly different effects on the uterine expression of BDNF and its receptors. Estradiol treatment significantly increased the uterine expression of mature BDNF (>6-fold), pro-BDNF (>5-fold) and NGFR (5-fold) when compared with the other treatments. While neither NTRK2 nor SORT1 were affected by ovarian hormones, continued daily exposure to estradiol increased mature BDNF which would lead to the induction of the BDNF-NTRK2 pathways, without affecting NTRK2 levels.

The neurotrophins are a complex network, and regulation of BDNF and NGFR by estrogen in the uterus can impact many BDNF pathways including angiogenesis (Kermani et al., 2005; Nakamura et al., 2006), cellular proliferation (Tervonen et al., 2006; Kawamura et al., 2010), adhesion (Zhou et al., 1997; Douma et al., 2004; Geiger and Peeper, 2007) and resistance to apoptosis (Douma et al., 2004; Wang et al., 2005; Geiger and Peeper, 2007; Kawamura et al., 2010; Li et al., 2011). Here, we also demonstrated the effect of estrogen on pro-BDNF in the uterus. The precise function of each BDNF isoform is only beginning to be elucidated, but generally pro-BDNF counteracts the effects of mature BDNF, providing another level of regulation for the powerful pathways activated by BDNF. We have shown a temporal effect to the hormonal regulation of NGFR in the cycling uterus, and highlighted the differential spatial localization of NGFR in response to ovarian hormones. The neurotrophins are involved in reproductive pathologies (Borghese et al., 2010; Browne et al., 2012; Bao et al., 2013; Barcena de Arellano et al., 2013), and physiological processes (Kawamura et al., 2009, 2011; Kerr et al., 2009; Dorfman et al., 2011, 2014; Shoae-Hassani et al., 2011; Nonet al., 2012). Although little is known about the functions of BDNF and its receptors within the reproductive

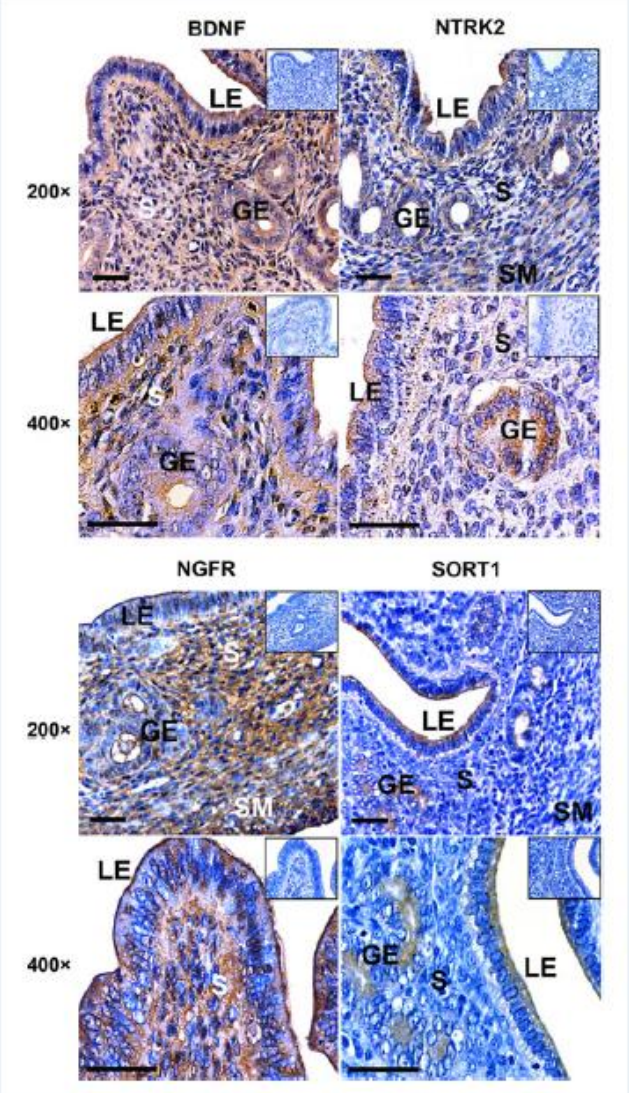


Figure 6 BDNF and receptor localization in the hormone replacement mouse uterus. Uterine localization of BDNF and its receptors in ovariectomized mice given hormone supplementation according to Fig. 1. BDNF, and NTRK2 were located in the luminal epithelium, glandular epithelium, stroma, and smooth muscle in the mouse uterus of all treatment groups (representative images from mice treated with estradiol). NGFR was found in all uterine cell types, but as in the cycling mice its localization was dependent on whether mice were exposed to estrogen or progesterone (see Fig. 4) (representative images from mice treated with estradiol). SORT1 was located on the apical side of the glandular epithelium, and the luminal epithelium in the uteri of mice in all treatment groups (representative images from mice treated with progesterone). Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200, ×400. Scale bar = 50 μm. GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle.

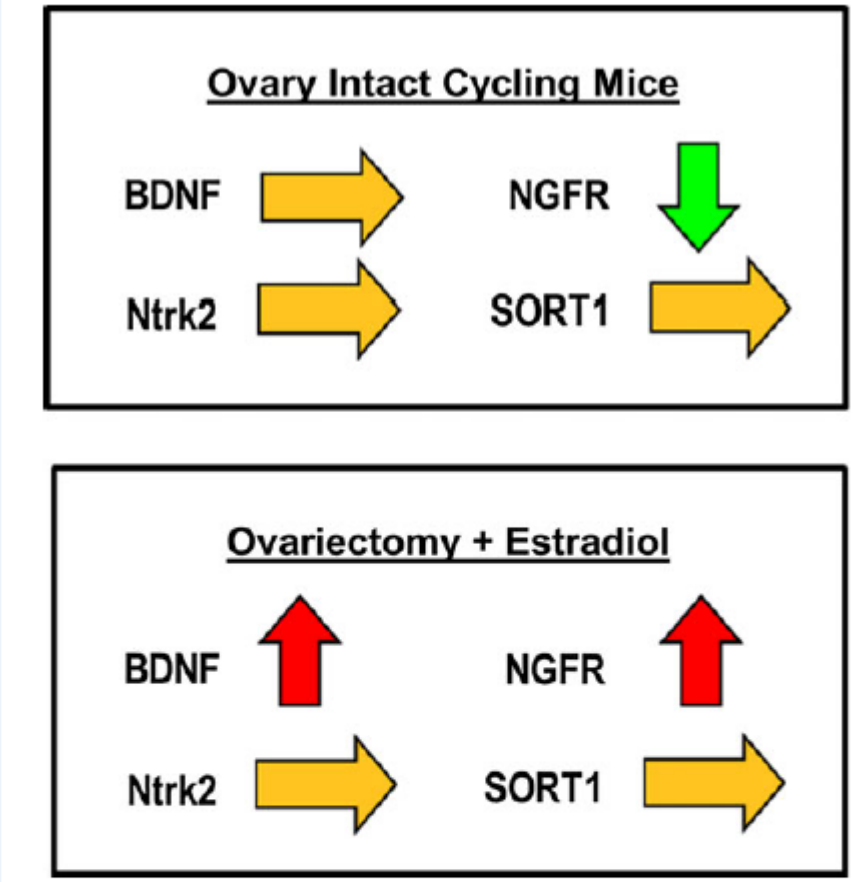


Figure 7 Contrasting uterine BDNF and receptor expression in cycling mice versus ovariectomy + estradiol replacement. A summary of the uterine expression of BDNF, NTRK2, NGFR and SORT1 in the uterus under physiological conditions (hormone exposure during the estrous cycle) when compared with mice undergoing daily estradiol replacement according to the regimen in Fig. 1 (E2 group). Green arrow: NGFR significantly decreases over the estrous cycle, under physiological conditions. Red arrow: BDNF and NGFR are significantly increased by daily estrogen exposure in ovariectomized mice. Yellow arrow: no change over the estrous cycle or treatment groups.

system, they are poised to participate in many aspects of reproductive physiology and pathology. The results of this study implicate estrogen in the uterine up-regulation of BDNF and NGFR, and highlight the differing effect of hormone exposure during the estrous cycle versus estradiol replacement after ovariectomy on neurotrophin expression. Sustained estrogen exposure, as seen in estrogen-dependent disease, may tip the neurotrophin balance and inappropriately activate pathways important in the disease pathophysiology.

Supplementary data

Supplementary data areavailable at http://humrep.oxfordjournals.org/. *****SEE APPENDIX I*****

3.7 Acknowledgments

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Authors' roles

All authors contributed to the study concept and design; drafted and critically revised the manuscript; and provided final approval of the version to be published. J.M.W. acquired, analyzed and interpreted the data.

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Conflict of interest

The authors do not have any conflicts of interest to declare.

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Chapter 4

Assessing Brain-Derived Neurotrophic Factor as a Novel Clinical Marker of Endometriosis

This article will be submitted for publication, 2015.

Unpublished Manuscript:

Wessels JM, Kay, VR, Leyland NA, Agarwal SK, and Foster WG (2015). Brain-derived Neurotrophic Factor is a Novel Clinical Marker of Endometriosis. Unpublished, 2015.

4.1: Chapter Introduction

In women with endometriosis BDNF expression in the eutopic endometrium has been shown to be significantly elevated compared to women without endometriosis by a recent proteomics study (Browne et al., 2012). Further, another preliminary study found that women with endometriosis had elevated plasma BDNF as compared with healthy, asymptomatic women, which fell after surgical removal of the lesions. (Giannini et al., 2010). Taken together, these studies report the dysregulation of BDNF in both the eutopic endometrium and circulation of women with endometriosis, and suggest that BDNF might be a useful non-invasive indicator of disease and response to treatment.

Therefore, the final objective of this Ph.D. thesis was to quantify circulating BDNF and other putative biomarkers of endometriosis including CA-125 and CRP in the plasma of women with and without endometriosis and assess their suitability as clinical markers of disease.

4.2: Article

Title: **Assessing Brain-Derived Neurotrophic Factor as a Novel Clinical Marker of Endometriosis**

Running Title: BDNF as a Clinical Marker of Endometriosis

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Keywords: Biomarker, CA-125, C-reactive protein, Endometriosis, Neurotrophins.

Number of figures and tables: 5

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4.3: Abstract

Objective: To evaluate novel clinical markers of endometriosis including the neurotrophins nerve growth factor (NGF), neurotrophin 4/5 (NT4/5), and brain-derived neurotrophic factor (BDNF), and compare them to other putative markers cancer antigen 125 (CA-125), and C-reactive protein (CRP) previously reported in the literature.

Design: Prospective study.

Setting: University Hospital.

Patients: 138 women were prospectively and consecutively recruited between April 2011 and April 2015 into the study (cases: women undergoing surgery for endometriosis, N=96; controls: benign gynecological surgery, N=24 combined with healthy women, no history of pelvic pain not undergoing surgery, N=18).

Intervention: Peripheral blood collected from cubital vein, gynecological and demographic information collected by survey, eutopic biopsy performed by pipelle in women undergoing laparoscopy.

Main Outcome Measures: Circulating concentrations of BDNF, NGF, NT4/5, CA-125 and CRP were quantified by ELISA.

Results: Plasma concentrations of BDNF were significantly greater (P=0.018) in women with endometriosis (1,091.9 pg/mL (640.4-1683.1); N=68, untreated) than controls (731.4 pg/mL (352.1-1176.2); N=36), whereas circulating NGF, NT4/5, CA-125, and CRP were not different. When the putative markers were assessed for their ability to differentiate between women with rAFS Stage 1&2, or 3&4 disease and controls, BDNF was the only marker able to identify the often clinically invisible Stage 1&2 disease, with a sensitivity and specificity of 91.7% and 69.4% respectively using an arbitrary cut-off value of 1,000 pg/mL. We also demonstrated that circulating BDNF in women with endometriosis who were receiving hormonal treatment (ovarian suppression) for disease was equivalent to circulating BDNF in the control group. This suggests that BDNF may also be a useful clinical tool to monitor patient response to treatment.

Conclusion: Plasma BDNF is a potentially useful clinical marker of endometriosis that is superior to NGF, NT4/5, CA-125, and CRP.

4.4: Introduction

Endometriosis is a chronic gynecological disease of unknown etiology characterized by the presence of endometrial fragments at ectopic locations (Rogers et al. 2009, Giudice 2010). It affects approximately 10% of women of reproductive age from all ethnicities, and is a major cause of severe pelvic pain, suffering, infertility, and hysterectomy (Eskenazi and Warner 1997, Cramer and Missmer 2002, Giudice 2010, Nnoaham et al. 2011). In the absence of a suitable diagnostic marker the interval between onset of symptoms of endometriosis and confirmed diagnosis by laparoscopy is 11.7 years in the U.S. (Ballard et

al. 2006). Lost time from work, costly medical interventions and surgical procedures all contribute to endometriosis being one of the largest healthcare expenditures with the annual cost of treatment and patient care reaching approximately $69.4 billion in the U.S. (Gao et al. 2006, Simoens et al. 2007, Simoens et al. 2012, reviewed in Burney and Giudice, 2012) and $1.8 billion in Canada (Levy et al. 2011). Significantly more resources are spent on endometriosis than other chronic conditions (migraines, asthma, and Crohn's disease) (Simoens et al. 2007) and thus identification of a clinical marker of disease remains a top priority.

Emerging evidence suggests an important role for the neurotrophins, a family of growth factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3, (NT-3), and neurotrophin 4/5 (NT4/5), in uterine physiology (Wessels et al. 2014, Wessels et al. 2015) and endometrial pathology (Anger et al. 2007, Borghese et al. 2010, Browne et al. 2012, Zhang et al. 2012, Barcena de Arellano et al. 2013). Results of a small study suggested that women with endometriosis had elevated circulating BDNF concentrations compared to healthy controls, which decreased after surgical removal of lesions (Giannini et al. 2010). Subsequently, protein expression for BDNF and its high affinity receptor were found to be greater in the uterus of women with endometriosis compared to disease-free controls (Anger et al. 2007, Browne et al. 2012). Therefore, the objectives of this prospective case-control study were to assess the suitability of circulating concentrations of neurotrophins including BDNF, NGF, and NT4/5 as clinical markers of endometriosis and to contrast our results with other putative clinical markers of

endometriosis including cancer antigen 125 (CA-125), and C-reactive protein (CRP) in the same population of women. Herein we present the results of our interim analysis of the study data.

4.5: Materials and Methods

Study Participants: One hundred and thirty eight women were recruited and screened for inclusion in the study (***Figure 1***). One hundred and twenty women undergoing gynecological laparoscopy between April 2011 and April 2015 for pelvic pain thought to be due to endometriosis were prospectively and consecutively recruited. Of these, 96 were found to have endometriosis (cases, N=96) and 24 were diagnosed with other benign gynecological conditions (symptomatic controls, N=24). Eighteen women with no history of pelvic pain and not undergoing surgery were also recruited (asymptomatic controls, N=18). The study exclusion criteria were: individuals unable to provide consent, age under 18, or a diagnosis of adenomyosis in the control group (3/138). All participants completed demographics and gynecologic questionnaires from which menstrual cycle length, date of last menstruation, and pelvic pain (4 5-point questions, totaled out of /20) were determined. Menstrual cycle stage was determined by uterine biopsy for women undergoing surgery and using the date of last menstruation for those not undergoing surgery. During laparoscopic surgery women were categorized as a case or symptomatic control by a gynecological surgeon and the diagnoses were confirmed by pathology reports. The stage of endometriosis was determined by the surgeon during surgery according to the revised Classification of the American Society of Reproductive Medicine (rAFS) (American

Society for Reproductive Medicine 1997). This study was approved by the Research Ethics Board, McMaster University (IRB#06-064, 14-066-T), and all participants provided written informed consent prior to surgery.

Peripheral blood was collected from participants into plasma and serum separator tubes (BD Canada, Mississauga, ON, Canada) by a nurse at McMaster University Medical Centre. Serum was not collected from most asymptomatic controls (N=16), nor a few cases (N=11). Blood was placed on ice, transferred to the laboratory, centrifuged at 3,000 rpm, and approximately 200µl of plasma or serum was aliquoted into 1.8mL cryovials (Sarstedt, Montreal, QC, Canada) and frozen at -80°C.

BDNF Assay: Plasma samples were thawed at room temperature and BDNF concentrations were quantified in triplicate using the BDNF Emax immunoassay ELISA (Promega, Madison, WI, USA) following the manufacturer's protocol. Briefly, 96 well NUNC maxisorp plates (Fisher Scientific, Ottawa, ON, Canada) were coated with anti-human BDNF antibody overnight. Freshly thawed plasma samples were diluted 1:10 with the provided sample buffer. Following incubation the absorbance was read at 450nm within 30 minutes using the Biotek Synergy spectrophotometer (Fisher Scientific). The kit sensitivity was 15.6 pg/mL.

NGF and NT4/5 Assays: Serum samples were thawed at room temperature and circulating NGF was quantified in duplicate in neat serum using the Human β-NGF Mini ELISA

Development Kit (Peprotech, Rocky Hill, NJ, USA) following the manufacturer's protocol. Incubations for the sample and detection antibody were lengthened to 3 and 2.5 hours, respectively. The kit has a sensitivity of 16 pg/mL. NT4/5 was quantified in duplicate using the Human NT-4 ELISA (RayBiotech, Norcross, GA, USA) which has a sensitivity of 2 pg/mL. The plates were incubated with neat serum overnight at 4°C, and according to the manufacturer's protocol. ELISAs were read as above.

CA-125 and CRP Assays: Circulating CA-125 and CRP were quantified in duplicate using the Human CA-125/MUC16 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) and Human CRP ELISA (Life Technologies, Burlington, ON, Canada), following the manufacturer's protocols. Plasma samples were thawed at room temperature and diluted 1:3 (CA-125) or 1:4000 (CRP) with the diluent provided. The sensitivity of the CA-125 and CRP assays is 0.035 U/mL and 10 pg/mL respectively. ELISAs were read as above.

Data and Statistical Analysis: Patient demographics were compared between cases and controls by t-test, Mann-Whitney Rank Sum Test, or Chi-square (SigmaStat 3.5 Systat Software Inc., Chicago, IL, USA) and are presented in Table 1 as mean±SD, median (25-75% percentiles) or N, %. For demographics which differed significantly between cases and controls multiple logistic regression was carried out to determine if any of the factors were significantly associated with being classified as a case or control. Nine women were excluded from the study due to missing samples (2/9), non-detectable BDNF (1/9), a diagnosis of adenomyosis (3/9), or they were classified as a control but taking Lupron (3/9).

In order to increase the sample size of the control group, we combined asymptomatic women who were (N=6) and were not (N=12) on oral contraceptives after determining that there was no significant difference in circulating BDNF between these groups (Supplemental Figure 1A, P=0.174, in Appendix I). Symptomatic controls who were (N=2) and were not (N=16) on oral contraceptives were also combined (Supplemental Figure 1B, P=0.663, in Appendix I). Next, the concentrations of BDNF, CA-125, and CRP were compared between the symptomatic and asymptomatic control groups (Supplemental Figure 1C, D, E, in Appendix I) by t-test or Mann-Whitney Rank Sum Test and did not differ significantly (P=0.159; 0.950; 0.137 respectively). Therefore, the two control groups (symptomatic and asymptomatic) were combined into one control group for all subsequent analyses. We also performed a sub-analysis of our data by menstrual cycle phase (Supplemental Figure 2A, B in Appendix I), as prior studies have shown significantly greater circulating BDNF during the secretory phase as compared to the proliferative phase in healthy, cycling women (Begliuomini et al., 2007; Pluchino et al., 2009). In our cohort of women, there was no significant difference in circulating concentrations of BDNF between cycle phase in cases or controls, and thus the analyses were not stratified by menstrual cycle phase. Circulating BDNF, NGF, NT4/5, CA-125, and CRP concentrations were compared by Mann-Whitney Rank Sum Test (cases (all stages) versus controls), or Kruskal-Wallis One Way Analysis of Variance on Ranks (across stage of disease, and by treatment) using SigmaStat (Systat Software Inc.) and are presented as median (25-75% percentile). Receiver operating characteristic (ROC) curves were compiled for circulating

BDNF, NGF, NT4/5, CA-125, and CRP using the ROC macro in SigmaStat. A P value of <0.05 was considered statistically significant.

4.6: Results

Patient Characteristics: Of the women recruited to participate in this study (N=138), 120 underwent laparoscopic surgery from which 96 cases of endometriosis and 24 symptomatic controls (women experiencing pain due to other indications including: pelvic pain no diagnostic abnormality (3/24), benign cysts (4/24), uterine fibroids (5/24), adenomyosis (excluded, 3/24), chronic inflammation (3/24), PCOS (3/24), endometrial polyps (1/24), or epidermoid cyst (2/24)) were identified. Three women in the control group were receiving Lupron, and thus excluded from the study (diagnoses: PCOS (1), fibroids (1), chronic inflammation (1)). An additional group of women with no history of pelvic pain (asymptomatic) not undergoing surgery were recruited as healthy controls (N=18). After the exclusion of women with adenomyosis (3), controls on Lupron (3), the removal of incomplete samples (2), non-detects (1), and amalgamation of control groups the final study population was 129 women: 93 cases and 36 controls (***Figure 1***).

The average age of cases was significantly higher (P=0.001) than controls (34.7±7.0 vs. 29.9±8.5, respectively, Table 1), ethnicity (P=0.004), occupational status (P=0.017), and smoking status (P=0.031) differed between cases and controls. Self-reported pelvic pain was significantly higher in cases than controls (9/20 vs. 3/20, P=<0.001). Multiple logistic regression was conducted using ‘case’ or ‘control’ as the dependent variable and age,

ethnicity, occupational status, smoking status, and pain as independent variables to determine their effect on the dependent variable. In this model, only pain (P<0.001) remained significantly associated with being a case or control, while age (P=0.055), ethnicity (P=0.265), occupational status (P=0.461), and smoking status (P=0.879) were not.

Menstrual cycle stage, current medical therapies, age at first menstruation (12 (11-13 years) cases vs. 12 (12-13 years) controls; P=0.639), and duration of bleeding in days (6 (4-7) cases vs. 6 (5-7) controls, P=0.817) were not different between groups. Of the 93 cases, 68 had not received any hormone treatment in the three months preceding surgery (21 were using NSAIDS or narcotic analgesics to manage pain), and 25 were being treated for endometriosis (hormonal contraceptives (9/25) and Lupron (16/25)).

Neurotrophins, CA-125, CRP and Endometriosis: Our dataset was analyzed separately (univariate analysis) for each putative marker, first regardless of stage of disease or menstrual cycle stage. The median circulating concentration of BDNF in the plasma was significantly greater (P=0.018) in women with endometriosis (1,091.9 pg/mL (640.4-1683.1); N=68, untreated) than controls (731.4 pg/mL (352.1-1176.2); N=36) (***Figure 2a***). In order to determine if circulating concentrations of BDNF were affected by menstrual cycle phase, the data was re-analyzed by phase (menstrual, proliferative, secretory) in untreated cases and controls separately (Supplemental Figure 2A, B, in Appendix I). There was no significant effect of menstrual cycle phase on circulating BDNF in cases (P=0.648) or controls (P=0.460), and thus analyses are not stratified by cycle stage. Further, as pelvic

pain had been found to be significantly associated with being a ‘case’ or ‘control’ in our preliminary statistical analysis, the relationship between pelvic pain and each putative biomarker was determined by linear regression in untreated cases and controls. No significant association was observed for any of the markers (Supplemental Figure 3A-E, in Appendix I), and thus analyses are not stratified by pelvic pain. Finally, no association between circulating BDNF and age was observed using linear regression in cases and controls (Supplemental Figure 3F, in Appendix I).

Serum samples were unavailable for asymptomatic women and 11 cases. However, circulating NGF in the serum of the remaining subset of untreated cases (N=57) was 71.1 pg/mL (29.7-173.4) and was not significantly different (P=0.418) from a subset of controls (N=22) who had concentrations of 77.9 pg/mL (28.5-99.2) (***Figure 2b***). In the same subset, the median circulating NT4/5 in the serum was 7.9 pg/mL (3.8-20.1), which did not differ significantly (P=0.351) compared to women without endometriosis who had 5.2 pg/mL (0.3-24.0) (***Figure 2c***).

In women with endometriosis (N=68, untreated), the circulating concentration of CA-125 in the plasma was 7.8 U/mL (4.0-18.9), and was not significantly different (P=0.369) than women without endometriosis (N=36) who had concentrations of 7.0 U/mL (5.1-10.5) (***Figure 2d***). In the same group of women, circulating CRP did not differ (P=0.929) between cases (2.2 μg/mL (0.6-4.6)) and controls (3.1 μg/mL (0.5-3.8) (***Figure 2e***). ROC curves for each of the putative markers were generated (***Figure 2f***), and BDNF was found

to have the greatest area under the curve (0.64; P=0.017) compared to NGF (0.56; P=0.42), NT4/5 (0.57; P=0.35), CA-125 (0.55; P=0.37) and CRP (0.51; P=0.93).

Neurotrophins, CA-125, CRP and Stage of Disease: The relationship between circulating BDNF, NGF, NT4/5, CA-125, CRP and stage of disease in women not receiving treatment for endometriosis (***Figure 3***) was determined. Women with Stage 1&2 endometriosis had significantly elevated BDNF (P=0.028) compared to controls (1,178.6 pg/mL (1043.8-1433.8) vs. 731.4 pg/mL (352.1-1176.2), respectively; Stage 1 & 2, N=12; Controls, N=36) (***Figure 3a***). No significant difference in circulating BDNF was found for women with Stage 1&2 versus Stage 3&4 (1,178.6 pg/mL (1043.8-1433.8) Stage 1&2, N=12; vs. 1,076 pg/mL (593.7-1433.8) Stage 3&4; N=56, respectively), nor between women with Stage 3&4 disease versus the control group (1,076 pg/mL (593.7-1433.8) vs. 731.4 pg/mL (352.1-1176.2), respectively). NGF (***Figure 3b***) and NT4/5 (***Figure 3c***) were compared across stage of disease and did not differ significantly (P=0.619; P=0.463 respectively).

Circulating CA-125 was significantly increased in women with Stage 3&4 endometriosis versus women with Stage 1&2 disease (P=0.007) (9.2 U/mL (4.8-21.7) vs. 3.7 U/mL (2.5-7.3); N=56, 12 respectively; ***Figure 3d***). There were no significant differences between women with Stage 1&2 or 3&4 disease and controls. Nor were significant differences in CRP observed between women with Stage 1&2 or 3&4 disease and controls (3.8 (0.9-4.6), 1.8 (0.6-4.6), and 3.1 μg/mL (0.5-3.8), respectively; P=0.638; ***Figure 3e***).

ROC curves for BDNF, NGF, NT4/5, CA-125, and CRP were generated including women with Stage 1&2 disease (N=12) who were not receiving endometriosis treatment compared to controls (***Figure 3f***). BDNF had the greatest area under the curve (0.75; P=0.009) compared to NGF (0.54; P=0.76), NT4/5 (0.49; P=1.04), CA-125 (0.27; P=1.98) and CRP (0.59; P=0.34). Using an arbitrary cut-off value of 1,000 pg/mL, the sensitivity and specificity of BDNF as a biomarker of Stage 1&2 disease were 91.7% (CI 61.5-99.8%) and 69.4% (CI 51.9-83.7%) respectively.

Neurotrophins, CA-125, CRP and Endometriosis Treatment: The effect of treatment on circulating levels of putative endometriosis biomarkers was assessed (***Figure 4***). The treated group of women had Stage 1&2 (7/25) and Stage 3&4 (18/25) disease, and treatments included oral contraceptives (9/25) and Lupron (16/25). No significant difference (P=0.203) in the concentration of BDNF was observed between women on oral contraceptives and Lupron (Supplemental Figure 2C), thus they were grouped together and called the 'treated' group in all subsequent analyses. Women in the untreated group (N=68) were not receiving endometriosis treatment (47/68), or were only using NSAIDs (15/68), or narcotic analgesics (6/68) to manage pain. The untreated group consisted of women in Stage 1&2 (12/68) and Stage 3&4 (56/68). Of the five putative markers quantified, only BDNF (***Figure 4a***) demonstrated a significant difference between untreated and treated women with endometriosis, and controls (1,091.9 pg/mL (640.4-1683.1) vs. 729.1 pg/mL (439.7-1488.2) vs. 731.4 pg/mL (352.1-1176.2) respectively; P=0.025). No significant

difference in circulating BDNF was observed between women treated for endometriosis and controls (P=0.971). There was no effect of treatment on circulating concentrations of NGF (71.1 (29.7-173.4) vs. 103.8 (70.6-346.1) vs. 77.9 (28.5-99.2) pg/mL; P=0.060) (***Figure 4b***), NT4/5 (7.6 (3.8-20.0) vs. 3.5 (0.7-37.9) vs. 5.2 (0.3-24.0) pg/mL; P=0.395) (***Figure 4c***), CA-125 (7.8 (4.0-18.8) vs. 8.3 (5.7-11.5) vs. 7.0 (5.1-10.5) U/mL; P=0.634) (***Figure 4d***), or CRP (2.2 (0.6-4.6) vs. 2.6 (1.5-3.8) vs. 3.1 (0.5-3.8) μg/mL; P=0.898) (***Figure 4e***) between untreated, treated, and control women respectively.

4.7: Discussion

Results of the present study reveal that plasma BDNF concentrations are greater in the circulation of women with endometriosis, particularly those with Stage 1&2 disease, compared to a control group consisting of symptomatic (women with pelvic pain but not endometriosis) and asymptomatic (healthy) women. Moreover, we demonstrated that employing BDNF as a biomarker of Stage 1&2 disease using an arbitrary cut-off value of 1,000 pg/mL resulted in a test with high sensitivity 91.7% (CI 61.5-99.8%) and an acceptable specificity 69.4% (CI 51.9-83.7%). We also show that CA-125 is significantly elevated in women with Stage 3&4 endometriosis vs. women with Stage 1&2 disease.

In this study, we sought to compare BDNF to other neurotrophins including NGF and NT/4/5 and other previously studied putative markers of endometriosis CA-125 and CRP (May et al. 2010, Fassbender et al. 2013, Toor et al. 2014) as a single, relatively non-invasive marker of endometriosis. The putative markers were combined in a multiple

logistic regression analysis as a panel (data not shown), however BDNF alone proved more suitable. Of the markers described herein, BDNF was superior due to its ability to detect rAFS Stage 1&2 disease, which is often difficult to diagnose clinically, and because it was lower in women receiving ovarian suppressive therapies for endometriosis (oral contraceptives and Lupron) than in untreated women. Taken together, these data suggest that plasma BDNF might be a useful clinical marker of endometriosis and a clinical tool to monitor patient response to treatment. Furthermore, the inclusion of BDNF in a panel of endometriosis biomarkers might be warranted, and might help increase the ability of the panel to detect Stages 1&2 disease.

Overall, we found circulating concentrations of BDNF were significantly higher in women with endometriosis who were not receiving treatment versus the control group. We also observed that circulating BDNF was lower in women receiving ovarian suppression to treat endometriosis as compared to untreated women. We acknowledge that it is ideal to include a three-month hormone-free treatment period prior to study enrollment to eliminate potential confounding effects of ovarian suppression. However, we suggest that the inclusion of treated cases in the present study is an accurate reflection of the clinical reality. Our results are in accordance with and expand upon the findings of a prior study (Giannini et al. 2010), which showed a significant elevation in plasma BDNF in women with Stage 1&2 disease versus healthy controls, and a decrease in concentration after surgical removal of lesions. However the previous study did not explore the relationship between circulating BDNF in women with endometriosis compared to women with pelvic pain but without

endometriosis (symptomatic controls), and did not include women with Stage 3&4 disease. Another larger study of fertility patients revealed a link between presence of a BDNF (Met) single nucleotide polymorphism and increased severity of endometriosis (Stages 3&4) which was thought to contribute to endometriosis-associated infertility (Zhang et al. 2012). Based on our results indicating that BDNF is elevated in Stage 1&2 disease, we hypothesize that the circulating concentration of BDNF might more accurately reflect disease activity (number of red/black lesions). This would, perhaps in part, explain the large variation in circulating BDNF in women with Stage 3&4 disease, where adhesions and inactive lesions often predominate. Furthermore, a SNP in the BDNF gene, as was observed in the Zhang et al. 2012 study might result in an increased number of active lesions, and thus severity of endometriosis. Taken together, several studies have now identified a link between BDNF and endometriosis.

We propose that an ideal clinical marker of endometriosis would be measureable in blood, sensitive and specific in identifying patients with all stages of the disease, and decrease in response to medical and surgical therapies. Our results revealed that, of all the markers studied, only plasma BDNF concentrations were higher in untreated cases than treated cases. Although both BDNF and NT4/5 had previously been shown to be overexpressed in the eutopic endometrium of women with endometriosis versus controls (Browne et al. 2012), serum NT4/5 levels were not different between cases and controls in the present study. Thus, we propose that although neurotrophin family members are potentially important in the pathophysiology of endometriosis, only plasma BDNF shows promise as

a novel clinical marker of endometriosis. Moreover, our results suggest that measurement of plasma BDNF may have value as a marker of treatment response in endometriosis patients. A prospective analysis of circulating BDNF in untreated women with endometriosis seeking treatment should be undertaken along with validated pain and quality of life questionnaires to address the utility of BDNF as a marker of patient response to treatment.

The strengths of our study include the prospective case-control design, confirmation of endometriosis diagnosis by surgery and pathology, inclusion of a treated group of women with endometriosis, and assessment of potential confounders (pain, age, menstrual cycle phase, ethnicity, occupation, and smoking status). We also consider the inclusion of a clinically relevant control group (symptomatic) as a strength of the study. Upon initial analysis these women were not different from healthy asymptomatic controls and thus they were merged into a single control group for subsequent analyses. Furthermore, while two studies in healthy cycling women found a significant increase in circulating BDNF during the secretory phase (days 20-24) as compared to the proliferative phase (days 6-8) (Begluiomini et al., 2007; Pluchino et al., 2009), we did not observe any difference in BDNF concentration between phases of the menstrual cycle in our study population. Our diverging results are likely explained due to the fact that women were not recruited on specific cycle days into our study. As there was no difference between cycle phases, the data was not stratified by cycle phase. The ability to quantify BDNF on any cycle day is an advantage for a clinical marker, as it can be quantified on the day a woman presents to the

clinic, and not delayed. Although the results of the present study are encouraging, there are a number of important limitations. Specifically, as a tertiary care centre for endometriosis, the majority of our patient population presents with advanced stage disease and thus the sample size for Stage 1&2 endometriosis is limited. Since there is generally little rationale to operate on women with Stage 1&2 disease we are restricted to incidental findings of endometriosis in women undergoing laparoscopy for other indications. Hence, recruitment of women with Stage 1&2 disease remains a challenge and may be best addressed through multi-site investigations of novel clinical markers. Another potential limitation is that our asymptomatic controls did not undergo surgery to rule out a diagnosis of endometriosis. However, if any of the asymptomatic controls were to have endometriosis, our results would be biased towards the null hypothesis; that no difference in circulating BDNF exists between women with and without endometriosis. Thus, we are confident in including these women in our study. Finally, the results of this study pertain to a particular study population, and thus our results need to be independently validated. Replication of this study at another, larger, institution will add external validity.

In conclusion, plasma BDNF is superior to NGF, NT4/5, CA-125 and CRP as a single, relatively non-invasive marker of endometriosis. Further, BDNF has promising sensitivity 91.7% and specificity 69.4% for detecting Stage 1&2 endometriosis, and may also provide an indicator of patient response to treatment.

Author Contributions: Authors contributed to study conception (W.G.F., N.A.L., and S.K.A.), design (W.G.F., J.M.W., N.A.L., S.K.A.), acquisition and analysis of data (J.M.W. and V.R.K.). All authors contributed to interpretation of the data, drafting and critically revising the manuscript.

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4.10: Figure Legends

Figure 1. Study Design. One hundred and thirty eight women were prospectively and consecutively recruited to participate in the study. Gynecological laparoscopy was performed on 120 women, from which a group of 96 women with endometriosis and 24 symptomatic controls were derived. An additional 18 healthy women who were not undergoing surgery were recruited as asymptomatic controls. After application of the exclusion criteria 93 cases, 18 symptomatic controls, and 18 asymptomatic controls remained. Of the 93 cases, 68 were not receiving treatment for endometriosis or were only

managing their pain symptoms, while 25 were receiving treatment for endometriosis including oral contraceptives and Lupron. The putative biomarkers of endometriosis were statistically compared between the symptomatic and asymptomatic controls, and did not differ. Thus, the control groups were combined (N=36) for all subsequent analyses.

Figure 2. Putative Biomarkers of Endometriosis. The circulating concentration of BDNF in the plasma was significantly elevated (P=0.018) in women with all stages of endometriosis who were not receiving hormonal treatment or Lupron (N=68) compared to women without endometriosis (N=36) (A). Neither circulating NGF (B) nor NT4/5 (C) differed significantly between a subgroup of cases (N=57) and controls (N=22). Circulating CA-125 (D) and CRP (E) were quantified in the same women as BDNF. Neither CA-125 nor CRP differed between cases and controls. Receiver operating characteristic (ROC) curves for BDNF, NGF, NT4/5, CA-125, and CRP were generated (F), and BDNF had the greatest area ('A') under the curve (0.64; P=0.017) as compared to NGF (0.56; P=0.42), NT4/5 (0.57; P=0.35), CA-125 (0.55; P=0.37) and CRP (0.51; P=0.93). Statistical significance was assessed using the Mann-Whitney U test with a P value <0.05 considered statistically significant, denoted by an asterisk (*) above the graph. Whiskers on the box plots represent the 10th and 90th percentiles while the lower limit of the box is the 25th percentile, and upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles respectively.

Figure 3. Neurotrophins, CA-125, CRP and Stage of Disease. Women with Stage 1&2 endometriosis (N=12) who were not receiving treatment had significantly elevated BDNF (P=0.028) as compared to controls (N=36) (A). There were no significant differences between women with Stage 1&2 versus Stage 3&4 disease (N=56), nor between women with Stage 3&4 disease versus controls. No significant difference in circulating NGF (B) nor NT4/5 (C) was observed between groups in a subset (Control=22, 1&2=9, 3&4=48). Circulating CA-125 was significantly increased (P=0.007) in women with Stage 3&4 endometriosis as compared to those with Stage 1&2 disease (D). No significant difference in CRP was seen between women with Stage 1&2 or 3&4 disease and controls (E). Receiver operating characteristic (ROC) curves for BDNF, NGF, NT4/5, CA-125, and CRP were generated for women with Stage 1&2 disease not receiving treatment for endometriosis (Stage 1&2; N=12) versus controls (N=36) (F), and BDNF had the greatest area ('A') under the curve (0.75; P=0.009) compared to NGF (0.54; P=0.76), NT4/5 (0.49; P=1.04), CA-125 (0.27; P=1.98) and CRP (0.59; P=0.34). Using an arbitrary cut-off value of 1,000 pg/mL, the sensitivity and specificity of BDNF as a biomarker of Stage 1&2 disease were 91.7% (CI 61.5-99.8%) and 69.4% (CI 51.9-83.7%) respectively. Statistical significance was assessed using the Kruskal-Wallis one-way ANOVA on ranks test with a P value <0.05 considered statistically significant, denoted by an asterisk (*) above the graph. Tukey's test was employed for *post hoc* testing. Whiskers on the box plots represent the 10th and 90th percentiles while the lower limit of the box is the 25th percentile, and upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles respectively.

Figure 4. Neurotrophins, CA-125, CRP and Endometriosis Treatment. Women in the untreated group (N=68) were not receiving endometriosis treatment whereas those in the treated group (N=25) were on oral contraceptives or Lupron. Circulating BDNF (A) was significantly elevated (P=0.025) in women with endometriosis who were not receiving treatment compared with women receiving treatment and controls (N=36). There was no significant difference in NGF (B), NT4/5 (C), CA-125 (D) or CRP (E) across the groups. Statistical significance was assessed using the Kruskal-Wallis one-way ANOVA on ranks test with a P value <0.05 considered statistically significant, denoted by an asterisk (*) above the graph. Tukey's test was employed for post hoc testing. Whiskers on the box plots represent the 10th and 90th percentiles while the lower limit of the box is the 25th percentile, and upper limit is the 75th percentile. The line within the box is the median of the data. Dots below or above the box plots are the 5th and 95th percentiles respectively.

4.11: Tables

Table 1. Patient characteristics of women with and without endometriosis. NSAID: non-steroidal anti-inflammatory drug, SD: standard deviation.

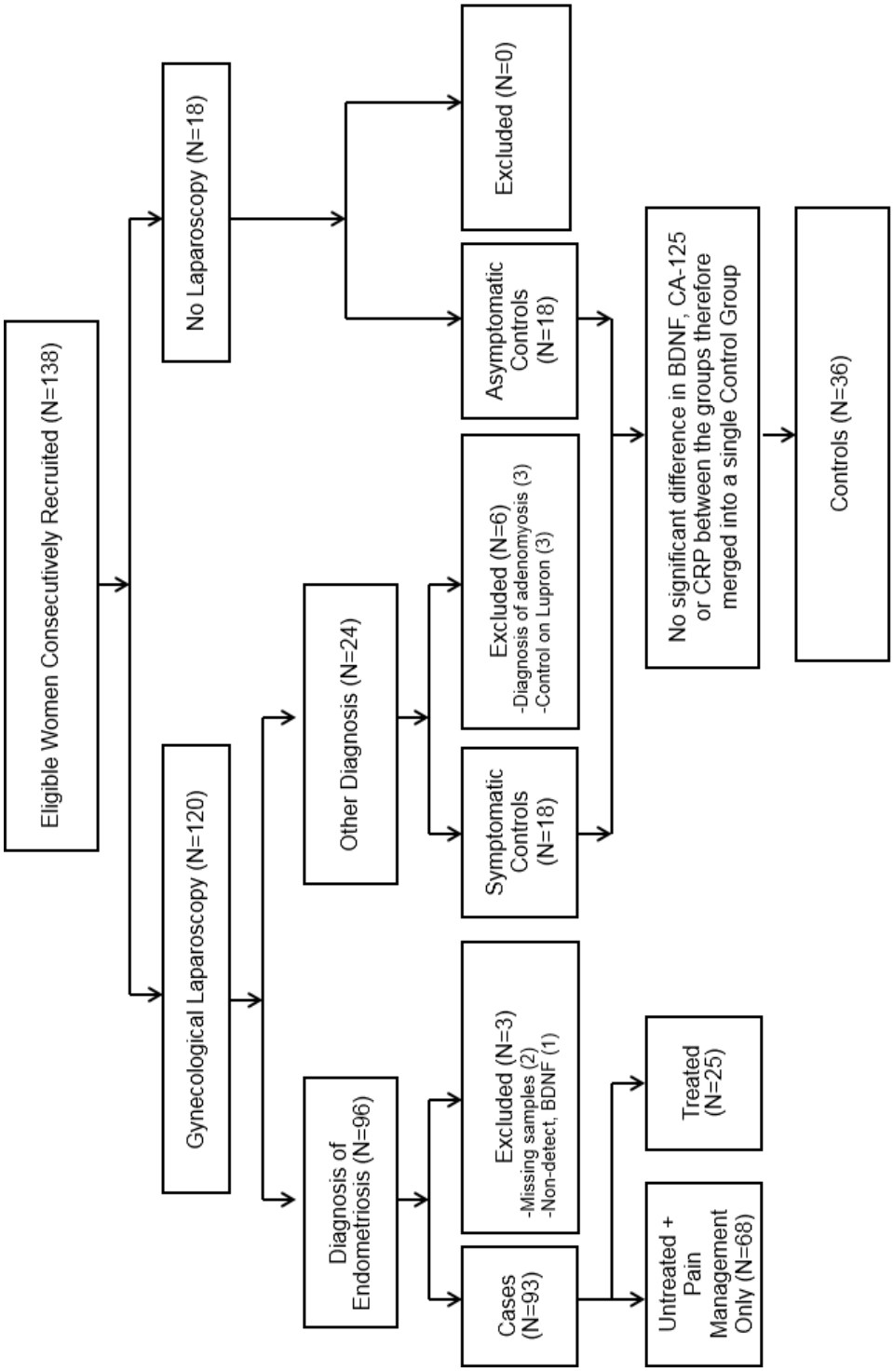
Characteristic	Control	Case	P
	N=36	N=93	
Age (Years)			
Mean±SD	29.9±8.5	34.7±7.0	*0.001*
Ethnicity (N, %)			
Caucasian	28 (78)	68 (73)	*0.004*
Asian	7 (19)	4 (4)	
Black	0 (0)	5 (5)	
Unknown	1 (3)	16 (17)	
Occupational Status (N, %)			
Employed	16 (44)	53 (57)	*0.017*
Unemployed	1 (3)	1 (1)	
Other	15 (42)	12 (13)	
Unknown	4 (11)	27 (29)	
Smoking Status (N, %)			
Non-Smoker	34 (94)	70 (75)	*0.031*
Smoker, <20 cigarettes/day	2 (6)	10 (11)	
Unknown	0 (0)	13 (14)	
Age at First Menstruation (Years)			
Median (25-75%)	12 (12-13)	12 (11-13)	0.639
Duration of Bleeding (Days)			
Median (25-75%)	6 (5-7)	6 (4-7)	0.817
Menstrual Cycle Stage (N, %)			
Menstrual	5 (14)	13 (14)	
Proliferative	9 (25)	19 (20)	0.348
Secretory	12 (33)	20 (22)	
Unknown	2 (6)	16 (17)	
Ovarian Suppression	8 (22)	25 (27)	
Pelvic Pain (Self-report, 0-20)			
Median (25-75%)	3 (2-8)	9 (6-11)	*<0.001*

Table 1. Continued

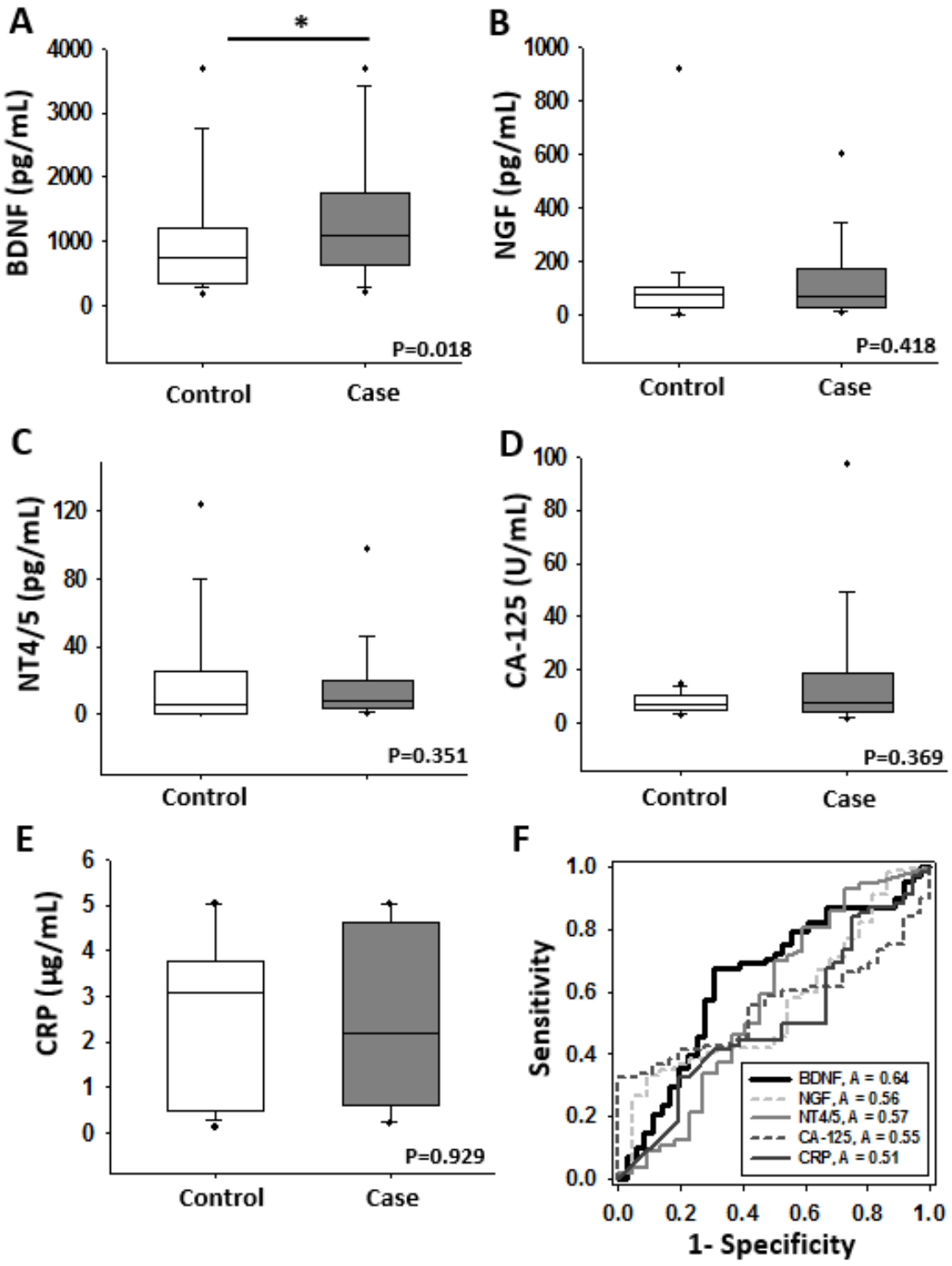
Current Medical Therapies (N, %)			
Hormonal Contraceptives	8 (22)	9 (10)	0.176
Lupron	0 (0)	16 (17)	
NSAID	2 (5)	15 (16)	
Narcotic Analgesic	1 (3)	6 (6)	
None/Other	25 (70)	47 (51)	
Stage of Endometriosis (N, %)			
Minimal, 1	0 (0)	10 (11)	N/A
Mild, 2	0 (0)	9 (10)	
Moderate, 3	0 (0)	10 (11)	
Severe, 4	0 (0)	64 (68)	

4.12: Figures

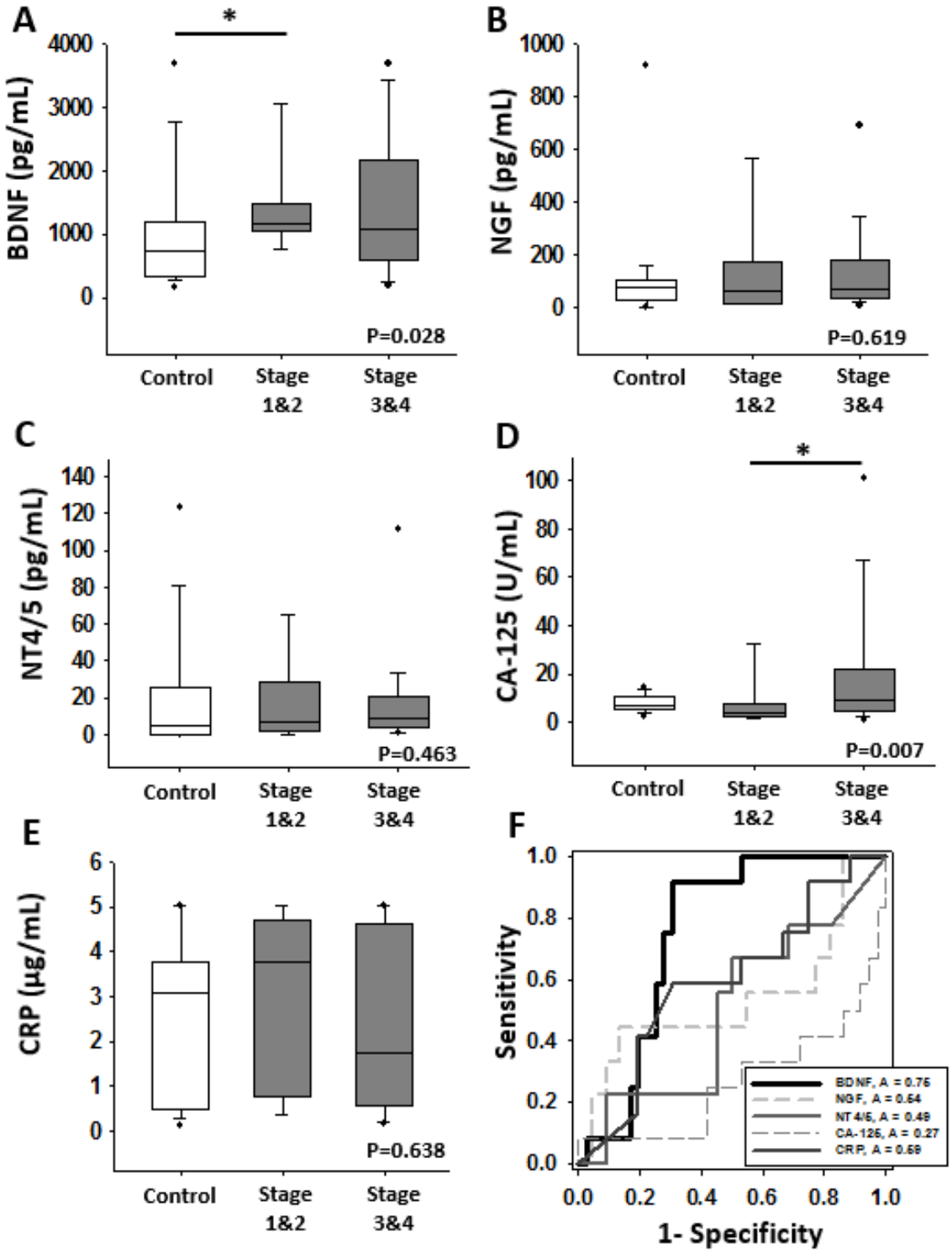
Wessels et al., Figure 1



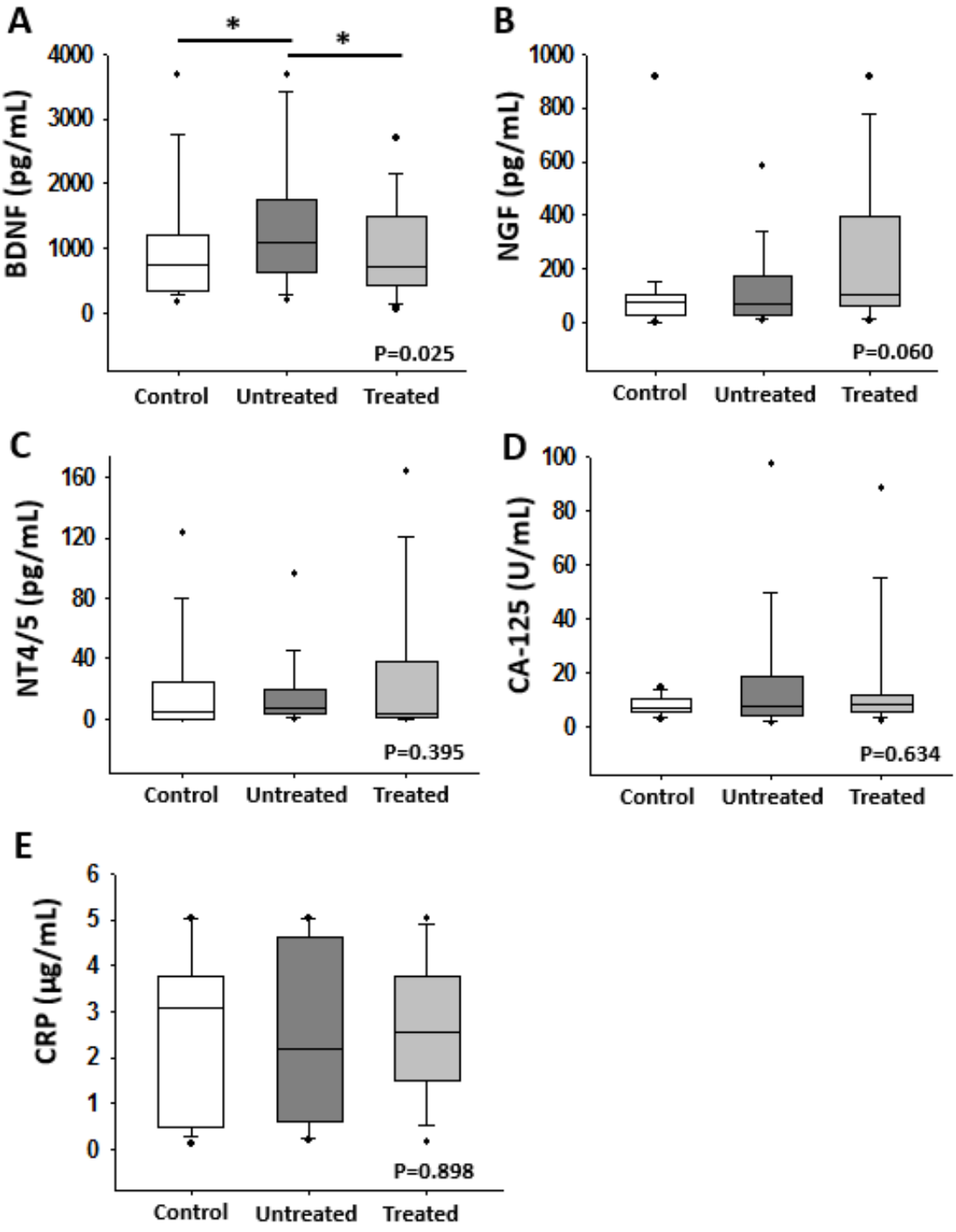
Wessels et al., Figure 2



Wessels et al., Figure 3



Wessels et al., Figure 4



Chapter 5

5.1: Discussion

Collectively the preceding three chapters of this Ph.D. thesis form a coherent and substantial body of work that advances our knowledge of uterine neurotrophins and endometriosis. In the second chapter the expression of BDNF and NTRK2 in the uterus of six mammalian species, including two that menstruate and four that do not, is established. Subsequently the upregulation of the expression of BDNF and its low affinity receptor NGFR in the murine uterus was demonstrated to be controlled by estradiol. Finally, because ectopic endometrial cells are exposed to excess estradiol in women with endometriosis, circulating BDNF was assessed for its ability to differentiate between women with and without endometriosis. Plasma BDNF concentrations were greater in women with endometriosis, particularly those with Stage I and II disease. Treatment with ovarian suppression therapies reduced circulating BDNF concentrations, and therefore might provide an opportunity to monitor patient response to endometriosis treatment. When compared with other putative biomarkers of endometriosis, including NGF, NT4/5, CA-125 and CRP, BDNF appears to be superior; as a marker of disease, particularly Stage I and II, and for its potential to monitor response to treatment.

The results of a recent systematic review suggested that six of the nine highest quality studies as assessed by the QUADAS criteria, identified endometriosis biomarkers relating to nerve fibre growth and cell cycle control (May et al., 2011). Despite this review, and reports of increased BDNF expression in the eutopic endometrium (Browne et al., 2012)

and elevated circulating BDNF concentrations (Giannini et al., 2010) in women with endometriosis compared to controls, relatively little was known about the expression and function of BDNF and its receptors in the tissues of the reproductive system. As such, the purpose of this thesis was to describe BDNF expression and regulation in the uterus, and assess BDNF as a relatively non-invasive clinical marker of endometriosis. The studies contained herein were undertaken with the hypothesis that BDNF is an estrogen-regulated growth factor expressed by endometrial cells that will provide a novel, non-invasive clinical marker of endometriosis in women.

5.2: BDNF Expression in the Uterus

In chapters two and three we set out to lay the foundation for future studies on BDNF in the reproductive system by demonstrating the expression of BDNF and its receptors in the mammalian uterus, and establishing its regulation by estradiol. The data in Chapter 2 illustrates the conservation of BDNF and NTRK2 expression in the uterus of species that do (humans, and fulvous fruit bats), and do not (mice, rats, pigs, horses) menstruate, and highlights their mainly epithelial localization. Indeed, the coding region of each gene was highly conserved, uterine transcripts for both *Bdnf* and *Ntrk2* were detectable by real-time PCR, and both BDNF and its high affinity receptor were co-localized mainly in the luminal and glandular epithelium in all species examined. Furthermore, BDNF and NTRK2 isoforms in human uterine homogenates were demonstrated by Western blot, revealing predominantly pro- and mature BDNF (mBDNF), and a truncated NTRK2 receptor. Our

results highlighting the conservation of uterine BDNF and NTRK2 expression amongst mammalian species proposes that this ligand-receptor pair participates in aspects of uterine physiology that remain to be explored.

5.3: BDNF Regulation in the Uterus

Although BDNF and its high affinity receptor were expressed in the uterus, their uterine regulation was entirely unexplored. In Chapter 3, we demonstrated the regulation of BDNF and NGFR by estradiol in the mouse uterus. We chose to assess the uterine regulation of BDNF and its receptors by estrogen and progesterone because BDNF expression in the brain has been shown to be regulated by these hormones (Solum and Handa, 2002; Kaur et al., 2007; Meyer et al., 2012; reviewed in Pluchino et al., 2013). Considering the profound impact of estrogen and progesterone on uterine target cells, the objective of Chapter 3 was to determine whether the uterine expression of BDNF and its receptors could be modified by estradiol, progesterone, or a combination of both.

In order to assess the uterine regulation of BDNF and receptors by estradiol and progesterone, we conducted two *in vivo* experiments. The first experiment was designed to observe BDNF and its receptors over the murine estrous cycle, while the second aimed to manipulate hormone exposure in ovariectomized mice. The purpose of this was twofold. It allowed for comparison between the naturally cycling mice, and those exposed to only estrogen or progesterone, and also allowed for contrast between a uterine environment

dominated by estradiol, progesterone, or both hormones. Considering that NTRK2 fluctuates in the murine brain over the estrous cycle (Spencer et al., 2008), it was expected that BDNF and its receptors would respond in a similar manner in the uterus. Surprisingly, the expression of BDNF, NTRK2, and the BDNF co-receptor SORT1 were stable over the estrous cycle in intact mice, while NGFR expression decreased from proestrus to diestrus. Even more surprising was the contrast in BDNF and NGFR expression between intact versus ovariectomized mice exposed only to estradiol. In response to estradiol exposure, ovariectomized mice had significantly elevated uterine expression of pro-BDNF, mBDNF, and NGFR, while none of these factors fluctuated over the estrous cycle in intact mice. Further, in ovariectomized mice exposed to progesterone only, or estradiol combined with progesterone the expression of BDNF and NGFR was no different from control animals, suggesting that progesterone antagonizes the stimulatory effect of estradiol and stabilizes the expression of BDNF and NGFR in the uterus. Thus, under physiological conditions, the uterine expression of BDNF and its receptors is fairly stable. However, when uterine cells are exposed predominantly to estradiol, their expression of BDNF and its low affinity receptor NGFR is markedly enhanced.

Another interesting observation from Chapter 3 was that the endometrial localization of NGFR switched from primarily stromal cells during proestrus, to epithelial cells during diestrus. We suspect that this phenomenon can be attributed to the dominance of either estradiol or progesterone because stromal NGFR expression was seen in ovariectomized mice receiving estradiol, whereas epithelial NGFR expression was observed in

ovariectomized mice receiving progesterone. Thus, the uterine localization of NGFR is likely regulated by estrogen and progesterone. In the brain the expression of BDNF is spatially regulated (Solum and Handa, 2002), and while uterine BDNF was not spatially regulated, NGFR was. The compartmentalization of NGFR during one cycle phase as compared to another might serve to divert soluble BDNF towards its high affinity receptor, NTRK2, during the estrogen-dominated phases, while diverting soluble BDNF towards NGFR during progesterone-dominated diestrus. The tissue compartment specific regulation of BDNF would differentially regulate the pathways that BDNF is able to activate during the early phases of the estrous cycle as compared to the later phase.

Upon completion of the second and third chapters we had demonstrated the presence of BDNF, its high affinity receptor, NTRK2, its low affinity receptor, NGFR, and co-receptor SORT1 in the mammalian uterus, established the uterine regulation of BDNF and NGFR by estradiol, and documented a change in NGFR localization in response to estradiol over progesterone.

5.4: Function of BDNF in the Uterus

Although the function of BDNF in the uterus was not directly assessed in this thesis, we can begin to infer its function based on studies performed in other body systems combined with the results of our studies. In addition to its trophic action on neurons in the brain and nervous system, there is evidence to suggest that the interaction between BDNF and NTRK2 activates many pathways in non-uterine cell types that are necessary for

reproduction including cellular adhesion (Zhou et al., 1997; Geiger and Peeper, 2007), proliferation (Glass et al., 1991; Represa et al., 1993; Elkabes et al., 1996; Lawn et al., 2015), resistance to apoptosis (Douma et al., 2004; Wang et al., 2005; Geiger and Peeper, 2007; Kawamura et al., 2010; Nikoletopoulou et al., 2010; Li et al., 2011), and angiogenesis (Kim et al., 2004; Nakamura et al., 2006; reviewed in Kermani and Hempstead, 2007; Blais et al., 2013; Kilian et al., 2014; Lin et al., 2014b; Usui et al., 2014; Dalton et al., 2015).

Within the brain and nervous system the neurotrophin signalling network is complex. The neurotrophins are initially translated intracellularly as pro-neurotrophins which can be enzymatically cleaved into their mature forms by pro-protein convertases including furin (Seidah et al., 1996; Mowla et al., 2001). Alternately, the pro-forms of the neurotrophins can be released from the cell and undergo extracellular processing by plasmin (Wolf et al., 1993; Gray and Ellis, 2008), and matrix metalloproteinases (Lee et al., 2001; Hwang et al., 2005). They can be released from either a constitutive or regulated secretory pathway (Heymach et al., 1996). The neurotrophins elicit their trophic effects by signalling through the NTRK family, and NGFR. In addition to the NTRK family and NGFR receptor there is an emerging, yet lesser known, neurotrophin co-receptor SORT1. SORT1 was recently shown to interact with pro-neurotrophins in the brain and to control their release in either a constitutive or activity-dependent manner (reviewed in Nykjaer and Willnow 2012). It may also be involved in a complex intracellular trafficking network directing proteins to various fates: cell surface expression, secretion, endocytosis, or transport within the cell (reviewed in Nykjaer and Willnow 2012).

Both BDNF and its precursor are biologically active, with mBDNF preferentially binding NTRK2, and pro-BDNF binding NGFR (reviewed in Deinhardt and Chao, 2014). Additionally, the affinity of mBDNF for NTRK2 can be enhanced by receptor dimerization with NGFR, while the affinity of pro-BDNF for NGFR can be enhanced by receptor association with SORT1 (Bibel et al., 1999; Deinhardt and Chao, 2014). While the interaction between mBDNF and NTRK2 activates adhesion, proliferation, angiogenesis, and resistance to apoptosis, the interaction between pro-BDNF and NGFR activates antagonizing pathways (Figure 6). As several BDNF isoforms and each of its receptors are expressed in the uterus, they are likely serving as regulators of the same physiological pathways described in other studies.

Based on our results, we speculate that the pathways activated by BDNF during the proliferative phase of the menstrual cycle in women gradually switch during the secretory phase to antagonizing pathways, as progesterone is synthesized by the corpus luteum. In Chapter 3 we demonstrated that the enhanced expression of BDNF and NGFR, and the stromal sequestration of NGFR in the uterus was controlled by estradiol. We also know from Chapter 2 that BDNF and NTRK2 were mainly expressed by endometrial epithelial cells. Thus, in the proliferative phase of the menstrual cycle where there is a dominance of estradiol, it seems likely that BDNF interacts with NTRK2 in the epithelial cells, rather than with NGFR in the stromal cells. During the proliferative phase of the menstrual cycle

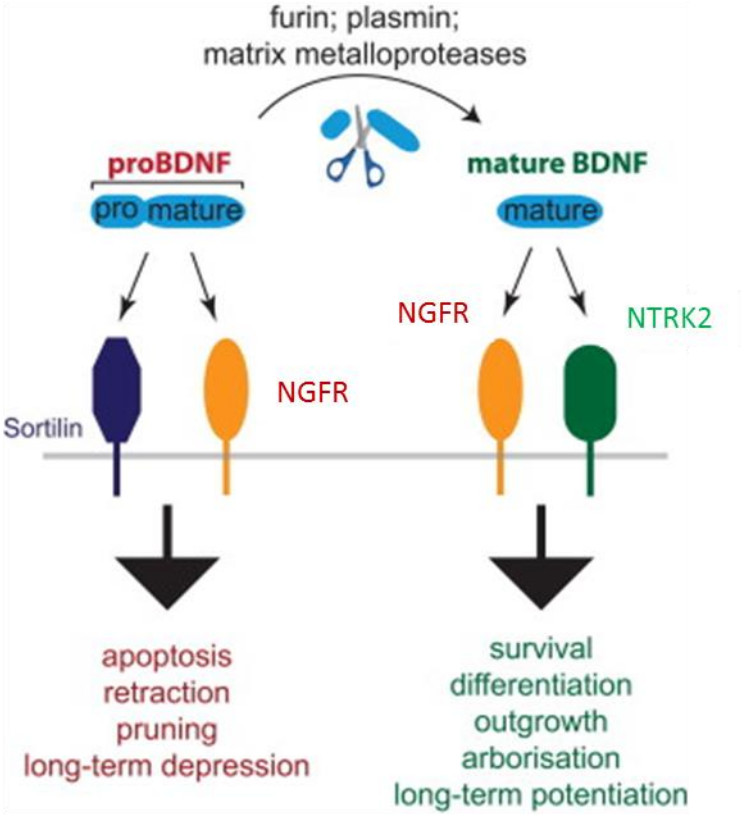


Figure 6: Opposing Effects of Pro- and Mature Brain-derived Neurotrophic Factor (BDNF) in Neurons. The interaction between pro-BDNF and nerve growth factor receptor (NGFR) and co-receptor sortilin (SORT1) activates signalling cascades resulting in apoptosis, neuronal retraction, dendritic pruning, and depression of neuronal activity. Conversely, the binding of mature BDNF to a receptor dimer consisting of its high affinity receptor neurotrophic tyrosine kinase receptor 2 (NTRK2) and NGFR activates opposing pathways that promote the survival, differentiation, growth, branching and long-term activation of neuronal activity. The dominance of one pathway over another is regulated in part by extracellular pro-protein convertases including furin, plasmin, and matrix metalloproteinases that cleave the off the pro- sequence, leaving mature BDNF to interact with receptors. As we demonstrate in chapter 2 and 3, each of these ligands and receptors are expressed in the uterus. We therefore suggest that they are able to activate antagonistic pathways in the uterus as well. The interaction between BDNF and NTRK2 has been demonstrated to induce adhesion, proliferation, angiogenesis, and resistance to apoptosis, while BDNF interacting with NGFR antagonizes these effects. Reprinted and modified from Deinhardt and Chao, 2014, with permission from Elsevier.

the endometrium is preparing for pregnancy. To achieve this, the luminal epithelial cells begin expressing adhesion factors to interact with blastocysts, cells proliferate to support implantation, and angiogenesis is occurring to allow for placentation and nutrient exchange. Ironically, these are the pathways induced by BDNF signalling through NTRK2. During the secretory phase, after ovulation has occurred, the corpus luteum is synthesizing progesterone. In the absence of blastocyst implantation progesterone gradually becomes the dominant reproductive hormone. In our second experiment (Chapter 3), progesterone attenuated the effect of estradiol on BDNF and NGFR expression, and the expression of NGFR was limited to the luminal and glandular epithelial cells. Therefore, during the secretory phase BDNF, NTRK2, NGFR, and SORT1 are likely co-expressed in the endometrial epithelial cells. The association between NGFR and SORT1 greatly enhances their affinity for pro-BDNF (Bibel et al., 1999; Deinhardt and Chao, 2014), and although pro-neurotrophins were originally considered inactive precursors, it is now believed that their biological action is to antagonize the actions elicited by their mature forms (Lee et al., 2001). We therefore postulate that in the absence of implantation, during the late secretory phase of the menstrual cycle pro-BDNF is preferentially binding to NGFR-SORT1 and activating pathways antagonistic to those activated by BDNF-NTRK2, which prepare the endometrium for menstruation. Proliferation, adhesion, and angiogenesis are attenuated in the endometrium, and apoptosis is initiated. We further speculate that the occurrence of pregnancy would rescue the endometrial lining from these fates, tipping the neurotrophin balance in the favour of BDNF-NTRK2 pathways.

5.5: BDNF as a Clinical Marker of Endometriosis

In the fourth chapter of this thesis we demonstrated that plasma BDNF concentrations were greater in women with endometriosis, particularly those with Stage I and II disease, as compared to controls. We also demonstrated that women undergoing endometriosis treatment with ovarian suppression therapies had reduced circulating BDNF concentrations when compared with women with endometriosis who were not receiving treatment. Finally, we compared BDNF with other putative biomarkers of endometriosis including NGF, NT4/5, CA-125 and CRP which were quantified in the same cohort of women. Out of the five markers, BDNF appeared to be superior due to its ability to indicate Stage I/II disease, the often clinically invisible stages of disease, with the highest sensitivity and specificity, and because it was the only putative marker examined that may provide an opportunity to monitor patient response to endometriosis treatment. In Chapter 4 we presented the first clinical study to critically assess BDNF as a marker of endometriosis.

After determining in Chapters 2 and 3 that BDNF and its receptors were positioned to participate in several of the major aspects of reproductive physiology we were impelled to know if they are similarly involved in endometrial pathologies, specifically endometriosis. As BDNF has mainly been studied in the nervous system, the literature has described low concentrations of circulating BDNF in patients with neurological disorders including Alzheimer's (Laske et al., 2006; Laske et al., 2007), Huntington's (Ciammola et al., 2007), Parkinson's (Ricci et al., 2010; Scalzo et al., 2010), autism (Taurines et al., 2014), schizophrenia (Toyooka et al., 2002; Akyol et al., 2015), depression (Karege et al., 2002;

Karege et al., 2005), bipolar disorder (Rabie et al., 2014; Piccinni et al., 2015), mood disorders (Polyakova et al., 2015), and eating disorders (Nakazato et al., 2003; Monteleone et al., 2005). Although mostly associated with neurological disorders, low concentrations of circulating BDNF have also been associated with cardiovascular disease (Fukushima et al., 2015; Kaess et al., 2015), impaired insulin function (Arentoft et al., 2009), type 2 diabetes (Krabbe et al., 2007), multiple sclerosis (Frota et al., 2009), and ulcerative colitis (Johansson et al., 2008). Conversely, there have been two small studies describing greater concentrations of circulating BDNF in patients with rheumatoid arthritis (Grimsholm et al., 2008), and fibromyalgia (Haas et al., 2010) as compared to healthy controls suggesting an association between inflammation, pain, and circulating BDNF. While the majority of diseases and conditions are associated with low concentrations of circulating BDNF, the fact that there are reports of two conditions (rheumatoid arthritis and fibromyalgia) in which plasma BDNF concentrations might be increased suggested that we document comorbidities in our clinical study, and stratify our data if necessary. To date, one preliminary study has quantified BDNF in the plasma of women with endometriosis, and found it to be higher in women with Stage I and II disease than healthy, asymptomatic women during the proliferative phase of the menstrual cycle (Giannini et al., 2010). As low concentrations of circulating BDNF have been postulated to provide a proxy of decreased BDNF expression in the brain, and a preliminary study supports increased quantities of BDNF in the plasma of women with endometriosis, we speculate that circulating BDNF might be proportional to the amount of endometrial tissue and thus active endometriotic lesions in women with endometriosis.

Given that BDNF expression in eutopic endometrial cells was influenced by excess estrogen in our animal model, that it was expressed in greater quantities in the eutopic endometrium of women with endometriosis (Browne et al., 2012), and that circulating BDNF was elevated in women with endometriosis but fell to concentrations similar to healthy, asymptomatic controls after surgical removal of the lesions (Giannini et al., 2010), we hypothesized that BDNF would be a useful, relatively non-invasive clinical marker of endometriosis and perhaps indicate response to treatment.

In the fourth chapter, we quantified circulating concentrations of three members of the neurotrophin family: BDNF, NGF, and NT4/5, and two previously reported putative markers of endometriosis CA-125 and CRP (reviewed in May et al., 2010). Notably, in our study the plasma concentration of BDNF was significantly higher in women with endometriosis than in our control group. By grouping women as cases or controls, and not sub-dividing by menstrual cycle phase or comorbidities we were likely biasing our results towards the null hypothesis; that there was no difference in circulating concentrations of BDNF between groups. However, when our analysis was performed we did observe significantly greater BDNF concentrations in women with endometriosis than in those without, validating our decision not to stratify our data and further suggesting that the difference in concentrations might in fact be widened by using more rigorous inclusion and analysis criteria in future studies. Our clinical study presented in Chapter 4 not only supports, but also significantly expands upon the results of Giannini et al., 2010. First, our

results suggest that circulating BDNF is higher in women with endometriosis irrespective of disease stage. Second, we included women with pelvic pain but without endometriosis (symptomatic women) in our control group because distinguishing between this group and women with disease is the more clinically relevant contrast. Third, in our study blood samples were drawn on whichever day of the menstrual cycle women happened to be on when they presented at surgery, as opposed to blood collected only during the proliferative phase as they had been in the Giannini et al., 2010 study. This is advantageous because it offers the possibility of assessing a woman for endometriosis the day she is at the clinic rather than scheduling an additional appointment during a specific phase of her menstrual cycle. Fourth, our study population was larger than that of the previous study, increasing the reliability of the contrasts we reported between cases and controls.

While medical imaging can occasionally be employed to detect the endometriomas and recto-vaginal nodules sometimes present in Stage III and IV disease, Stages I and II are often more difficult to detect clinically. Therefore, a tool able to identify Stage I and II endometriosis would be particularly clinically useful and relevant. Of the three putative biomarkers we assessed BDNF was the only marker able to identify Stage I/II of disease (sensitivity and specificity 91.7 and 69.4%, respectively) while CA-125 was a better predictor of Stage III/IV disease. Although we had shown that circulating BDNF was significantly elevated in cases of all stages as compared to controls, when we stratified the cases by Stage I/II versus Stage III/IV, no significant difference between women with Stage III/IV disease compared with the controls was seen. We speculate that instead of relating

to the stage of disease, circulating BDNF relates to disease activity. Stages I and II endometriosis generally consist of active lesion types (red, black lesions), as compared to white lesions and adhesions often associated with Stages III and IV. As such, women diagnosed with Stage IV endometriosis who have extensive adhesions (inactive), but no active lesions are not likely to have the same quantity of BDNF in their circulation as women with Stage I or II disease, including multiple red lesions. Lesion heterogeneity may, at least in part, explain the wide range of circulating BDNF in our cases diagnosed with Stage III and IV disease, and also explain why no significant difference was seen in circulating BDNF between women with Stage III/IV disease and controls. Thus, we propose that future studies should consider two groupings for women with endometriosis, the first by disease activity/burden and the second by stage. Although the effect of disease activity, lesion type, disease burden, location and number of lesions on circulating concentrations of BDNF has not been assessed, we suggest these factors are likely important to consider and should be assessed in future studies.

In addition to identifying Stage I and II endometriosis, a clinical marker capable of indicating patient response to treatment would be of great use. We quantified BDNF, NGF, NT4/5, CA-125, and CRP in the circulation of women with surgically confirmed endometriosis who were receiving medical therapies for endometriosis including Lupron and oral contraceptives, and statistically compared each biomarker across three groups: controls, cases untreated, and cases treated. While NGF, NT4/5, CA-125 and CRP remained stable across these groups, circulating BDNF concentrations in women with

endometriosis undergoing ovarian suppression to treat their disease were significantly lower than in women not undergoing treatment. In fact, the circulating BDNF concentration in treated cases was equivalent to circulating BDNF concentration in the control group. Taken together, this further supports an association between circulating BDNF concentrations and disease activity, and also suggests that of the three markers examined, BDNF is the only one that may also be a useful clinical tool to monitor patient response to treatment.

Upon completion of the fourth chapter a strong argument has been put forth supporting the use of plasma BDNF as a relatively non-invasive clinical marker of endometriosis over other putative markers CA-125 and CRP. Thus, BDNF is a novel clinical marker of endometriosis that may have the potential to alleviate some of the emotional and financial burdens of endometriosis. A non-invasive clinical marker of endometriosis is long overdue and greatly needed. It will allow for a more timely diagnosis of disease, prompt treatment, and it will reduce patient suffering, positively impacting the lives of millions of women who suffer from endometriosis world-wide.

5.6: Proposed Role of BDNF in the Pathophysiology of Endometriosis

Based on the studies conducted herein a model proposing a role for BDNF and its receptors in the pathophysiology of endometriosis has been developed (Figure 7). The model proposes that the dysregulation of BDNF in endometriotic lesions contributes to lesion

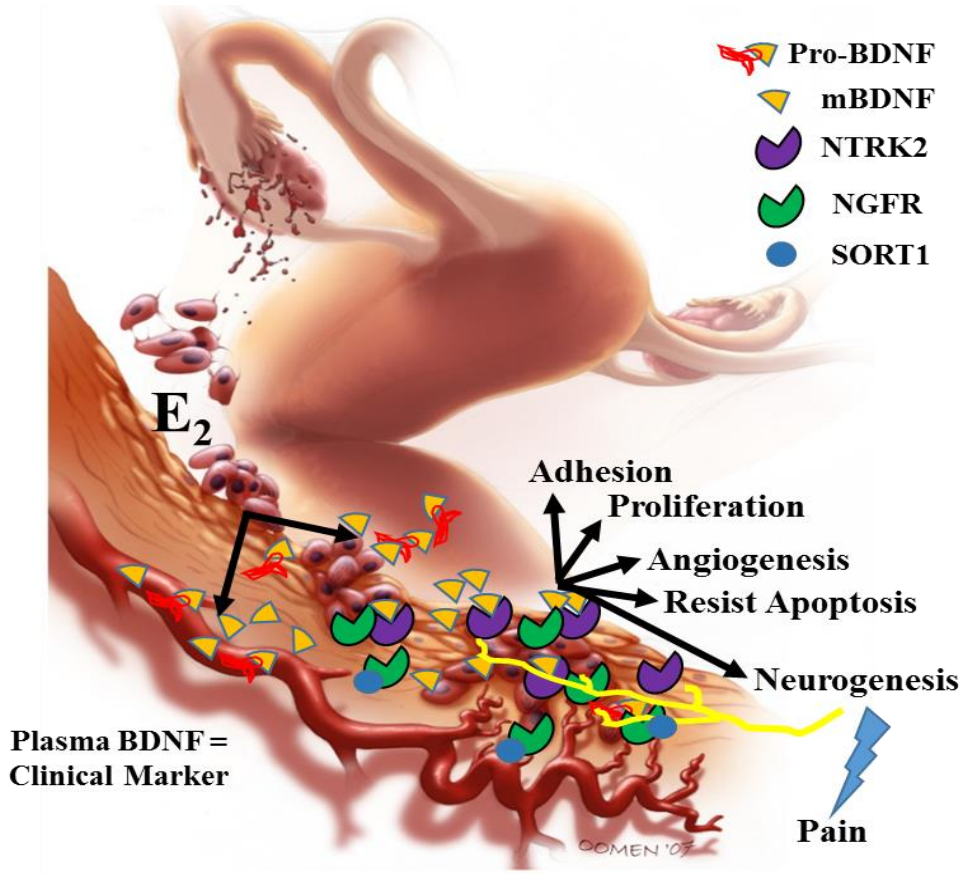


Figure 7: Proposed Role of Brain-derived Neurotrophic Factor (BDNF) in the Pathophysiology of Endometriosis. Endometrial cells are regurgitated into the peritoneal cavity during menses, and attach to the peritoneum. The cells invade the surrounding tissue and begin to express aromatase. The excess estradiol (E_2) synthesized via aromatase in the endometriotic lesions enhances the expression of pro-BDNF, mature BDNF (mBDNF), and nerve growth factor receptor (NGFR). As the endometriotic cells are unable to respond to progesterone, the cycle of E_2-driven BDNF expression continues unabated, accessing the systemic circulation where it can be quantified as a non-invasive clinical marker of endometriosis. Within the lesions, pro-BDNF is cleaved to mBDNF by peri-cellular pro-protein convertases including furin, plasmin, and matrix metalloproteinases. Dimerization of NGFR with neurotrophic tyrosine kinase receptor 2 (NTRK2) increases the affinity of NTRK2 for mBDNF, preferentially activating the adhesion, proliferation, angiogenesis and resistance to apoptosis pathways as opposed to the antagonistic pathways activated by pro-BDNF binding NGFR/sortilin (SORT1). Further, because of the participation of BDNF and NTRK2 in inducing neuronal growth, development, and maintenance in the nervous system, neurogenesis into the ectopic lesions is likely initiated, resulting in nerve fibre growth and endometriosis-associated pain. Reprinted and modified from Anger and Foster, 2008, under license from Frontiers in Bioscience.

establishment, growth and survival by activating many of the key pathways which are shown to be altered in women with endometriosis. We have demonstrated that BDNF is expressed by endometrial cells, particularly epithelial cells, and shown that its expression is significantly increased by estradiol. Moreover, our results suggest that progesterone counteracts this effect. Within the microenvironment of the endometriotic lesion there is excess estradiol (Huhtinen et al., 2012), and increasing evidence to suggest that the lesions are incapable of responding to progesterone (reviewed in Bulun et al., 2006). The dominance of estradiol in the ectopic lesions is a result of the presence of aromatase, the inability to convert estradiol to estrone (Zeitoun et al., 1998; Matsuzaki et al., 2006a; Delvoux et al., 2009), and the positive feedback loop driving inflammation, aromatase expression, and estradiol synthesis (Noble et al., 1997; Lindstrom and Bennett, 2004; Tamura et al., 2004; Attar et al., 2009). It thus stands to reason that the displaced endometrial cells that form endometriotic lesions respond to excess estradiol and lack of progesterone attenuation in a manner similar to eutopic endometrial cells. Extrapolating from the results of the study presented in Chapter 3 that highlighted the uterine regulation of BDNF and NGFR by estradiol, the abundance of estradiol in the endometriotic lesions is likely to increase the local expression of pro-BDNF, mBDNF, and NGFR. Previously, the association of NTRK2 with NGFR has been shown to enhance receptor affinity for mBDNF (Bibel et al., 1999). Further, the dominance of estradiol might also spatially restrict NGFR expression, mainly to the endometriotic stromal cells, as occurred in the murine uterus in Chapter 3. As we presented in Chapter 2, BDNF and NTRK2 are primarily localized in the endometrial epithelial cells. Therefore, we postulate that the enhanced

BDNF expression in endometriotic lesions in response to estradiol, increased receptor affinity of NTRK2 when dimerized with NGFR, and/or spatial sequestration of NGFR each serve to preferentially target BDNF to interact with NTRK2 over NGFR. Experimental evidence suggests that the interaction between BDNF and NTRK2 promotes many pathways that would support endometriotic lesion establishment, growth, and survival including cellular adhesion (Zhou et al., 1997; Geiger and Peeper, 2007), proliferation (Glass et al., 1991; Represa et al., 1993; Elkabes et al., 1996; Lawn et al., 2015), resistance to apoptosis (Douma et al., 2004; Wang et al., 2005; Geiger and Peeper, 2007; Kawamura et al., 2010; Li et al., 2011), and angiogenesis (Kim et al., 2004; Nakamura et al., 2006; reviewed in Kermani and Hempstead, 2007; Blais et al., 2013; Kilian et al., 2014; Lin et al., 2014b; Usui et al., 2014; Dalton et al., 2015). Thus, in addition to the trophic effects of estradiol, the active endometriotic lesions are very likely also under the influence of the neurotrophins.

Further evidence supporting the involvement of BDNF in the pathophysiology of endometriosis is gleaned from the results presented in Chapter 4. Women with endometriosis had greater circulating concentrations of BDNF, which were particularly associated with Stage I/II disease (abundant active lesions), as compared to controls. As BDNF is a soluble growth factor which can be released into the extracellular space (Wolf et al., 1993; Heymach et al., 1996; Gray and Ellis, 2008), it is plausible that the endometriotic cells release BDNF in response to estradiol, and that the BDNF is not only able to interact with surrounding cells in a paracrine manner, but gains access to the

systemic circulation, thus providing a clinical marker of disease. The physiological process of neovascularization is prominent in the developing lesions, and it would therefore be possible for BDNF to access the circulation. Further suggesting that the excess circulating BDNF in women with endometriosis may originate in the endometriotic lesions is the fact that circulating concentrations of BDNF fell after surgical removal of lesions in one study (Giannini et al., 2010), and in our study circulating BDNF concentrations were lower in women with endometriosis who were receiving treatment as compare to those who were not. The effect of medical therapies for endometriosis is to suppress ovarian estradiol, thus decreasing estradiol in the systemic circulation (Huhtinen et al., 2012). Although the endometriotic lesions are able to synthesize some estradiol locally, medical therapies deprive the endometriotic lesions of their systemic source of estradiol. As we demonstrated that BDNF expression is upregulated by estradiol in endometrial cells in Chapter 3, we suspect that restricting systemic estradiol in women receiving endometriosis treatment likely reduces expression of BDNF in the endometriotic lesions when compared to women with endometriosis not receiving any treatment. Thus, as the systemic estradiol is suppressed, BDNF concentrations should fall in parallel, which we allude to in our quantification of BDNF in the circulation of women receiving ovarian suppression to treat endometriosis as compared to those not receiving any treatment. Alternatively, endometriosis treatments suppress ovarian estradiol output which reduces BDNF originating from other sites. However, although the source(s) of circulating BDNF is presently unknown, women who have undergone surgical or medical therapies for

endometriosis have low concentrations of plasma BDNF as compared to women with untreated disease, proposing its endometriotic origin.

As BDNF is poised to affect many of the pathways central to endometriosis pathophysiology, it represents a novel therapeutic pathway. Unfortunately, because all of the current medical therapies for endometriosis suppress fertility by inhibiting endogenous hormone synthesis, women with endometriosis are forced to choose between their desire to manage disease-associated pain or their desire to try to become pregnant. If appropriately targeted, suppressing BDNF in the endometriotic lesions might be the first treatment for endometriosis that does not suppress fertility as a side effect. Indeed, a reduction in circulating BDNF was observed in a recent trial assessing the efficacy of melatonin as a treatment for endometriosis (Schwertner et al., 2013). In the double-blinded study, participants receiving melatonin had significantly lower pain scores, analgesic use, and circulating concentrations of BDNF than women in the placebo group, suggesting further study of this novel therapeutic avenue.

While there have been many factors and pathways associated with endometriosis, the neurotrophins, BDNF in particular, warrant further investigation. As the neurotrophin system is complex, it offers many opportunities to therapeutically manipulate these signalling pathways. For example, within the endometriotic lesion tipping the ratio of mBDNF towards pro-BDNF, inducing expression of a non-signalling truncated NTRK2

receptor, or enhancing the interaction between pro-BDNF and NGFR might each serve to enhance apoptosis of the lesion as opposed to promoting its survival.

Another intriguing link between the neurotrophins and endometriosis is their mutual association with nerve fibres. The primary clinical symptom of endometriosis is pelvic pain. While nerve fibres in the eutopic endometrium (Tokushige et al., 2006a; Tokushige et al., 2006b; Tokushige et al., 2007; Al-Jefout et al., 2007; Al-Jefout et al., 2009; Bokor et al., 2009; Aghaey Meibody et al., 2011; Elgafor El Sharkwy, 2013) and ectopic lesions (Tulandi et al., 1998, Al-Fozan et al., 2004; Kelm Junior et al., 2008; Wang et al., 2009a; Wang et al., 2009b, Tokushige et al., 2010; Zhang et al., 2010; Wang et al., 2011; McKinnon et al., 2012) have been reported in women with endometriosis, the current body of literature fails to adequately explain the link between nerve fibres and endometriosis. As the neurotrophins are potent neuronal growth factors, their involvement in endometriosis might be more extensive than simply supporting lesion growth and development; they might be the factors responsible for disease-associated pain. Indeed, neurite growth is modulated by BDNF in the rat uterus (Krizsan-Agbas et al., 2003), and the sensory innervation of the female murine mammary gland requires BDNF-NTRK2 signalling (Liu et al., 2012). In male mice, the lack of sensory innervation to the mammary gland is a result of the androgen-driven expression of a truncated NTRK2 receptor which inhibits the BDNF-NTRK2 pathways promoting neuronal growth (Liu et al., 2012). Taken together, these experiments suggest that the interaction between endometrial-cell derived BDNF and NTRK2 in the glandular epithelium of endometriotic lesions is likely capable of inducing

sensory innervation of the lesions, and that this process can be reversed by inhibiting BDNF-NTRK2 binding.

The medical therapies for endometriosis have been demonstrated to significantly reduce nerve fibre density in the endometrium and myometrium of afflicted women (Tokushige et al., 2008). Coincidentally, the expression of NTRK2 in deep-infiltrating endometriosis is reduced by GnRH agonist and oral progestin treatment (Matsuzaki et al., 2007). Combined with the results of the present thesis which indicate that BDNF is also likely to be similarly affected by ovarian suppression, we propose that the decline in BDNF and NTRK2 helps abate sensory innervation of the endometriotic lesions, and thus inhibits additional disease-associated pain.

Taken together, the results of the studies contained herein contribute to our understanding of the neurotrophins and endometriosis. Additionally, they advance our knowledge in both fields, and appropriately complement the existing literature. The results of these studies are supported by a scarce but growing body of literature associating the neurotrophins with endometriosis.

5.7: Strengths of the Thesis

There are a number of strengths to the studies contained within this thesis. All experiments were performed *in vivo*, either in experimental animals or on clinical samples collected from women of reproductive age at McMaster University Medical Centre.

5.7.1: Animal Experiments

Several of the major strengths of this thesis are due to the use of animal tissues to study reproductive phenomena. In the study presented in Chapter 2, six mammalian species including two that menstruate (humans and fulvous fruit bats) and four that do not menstruate (mice, rats, horses, and pigs) were explicitly chosen for a cross-species comparison to demonstrate the conserved nature of uterine neurotrophin expression. The importance of the comparison between species was twofold. First, the literature surrounding BDNF and NTRK2 expression in the mammalian uterus was incomplete, and a study demonstrating the presence of both ligand and receptor in the uterus was lacking. While some studies alluded to the expression of the ligand or receptor in the uterus (Krizsan-Agbas et al., 2003; Anger et al., 2007), another study was unable to detect uterine NTRK2 expression (Shibayama and Koizumi, 1996), perhaps due to the reagents available at the time. However, the majority of studies describing the non-neuronal distribution of BDNF and NTRK2 simply did not evaluate reproductive tissues or cell types (Yamamoto and Gurney, 1990; Kerschensteiner et al., 1999; Lommatzsch et al., 1999; Nakahashi et al., 2000; Noga et al., 2003; Rost et al., 2005; Hahn et al., 2006; Noga et al., 2008). Nevertheless, by demonstrating the presence of both ligand and receptor in six mammalian species, the expression of BDNF and NTRK2 in the mammalian uterus is no longer equivocal; it is a fact. Second, demonstrating the expression of BDNF and NTRK2 in the uterus of species that do and do not menstruate is another strength of the study presented in Chapter 2 because it adds a more thorough and comprehensive assessment of their

function within the uterus. Showing that uterine BDNF and NTRK2 are conserved, even between species that do and do not menstruate allows us to infer that their uterine function is related to reproduction as opposed to strictly menstruation. It also helps us to validate the use of animal models that do not menstruate, including mice, to examine physiological processes occurring in those that do menstruate, which may not be as easily accessed (bats, and women).

The experiments outlined in Chapter 3 were performed using mice as an animal model. Although it would have been ideal to describe the expression and regulation of neurotrophins in the human uterus, this would not have been possible except perhaps in primary cell culture. Additionally, there would have been several ethical and practical limitations to the study if it were performed in women. As an alternative, mice were selected. We believe our choice of model was appropriate and justified for the type of study we were conducting because we had previously demonstrated the conserved nature of neurotrophins in the mammalian uterus. Further, in the first experiment of Chapter 3 no experimental interventions were performed. Instead, the natural pattern of neurotrophin expression in the cycling mouse uterus was delineated. In order to assess the regulation of uterine BDNF and its receptors by the ovarian hormones, it was necessary to employ an animal model. In the second experiment of Chapter 3, we contrast the effect of estradiol and progesterone on BDNF, NTRK2, NGFR, and SORT1 expression in the ovariectomized mouse uterus. This choice of model is a strength of this thesis because it was practical, effective, and did not require a large number of animals to attain significance in our

statistical tests because the C57/Bl6 research mice are inbred and thus genetically identical. Realistically, this study could not have been performed in a more suitable model. Although primates are physiologically and genetically similar to humans, they would not have been a suitable model for this experiment due to their cost, their difficulty in handling, genetic variation, and inaccessibility at our research facility. Bats were not employed for similar reasoning. Our choice of employing mice to study the effect of estrogen and progesterone on the uterine expression of BDNF and its receptors is therefore a strength of this thesis.

5.7.2: Clinical Experiment

There are also several strengths of the clinical experiment which was undertaken to demonstrate the utility of BDNF as a novel clinical marker of endometriosis. The inclusion of symptomatic women with pelvic pain who underwent surgery and did not have evidence of endometriosis at surgery or at pathology as part of the control group is a major strength of the study. In our recent systematic review of putative endometriosis biomarkers, we identified that the majority of the papers reviewed (87.3%) that had scored greater than 6 on our modified QUADAS criteria used only healthy, asymptomatic women as controls in their studies (Toor et al., 2014). Considering that the primary clinical manifestation of endometriosis is pelvic pain, the most relevant control group would include women with pelvic pain, but without endometriosis. Besides, distinguishing between a woman with pelvic pain and endometriosis versus a woman with pelvic pain due to other indications is the more relevant clinical question, which we considered in our study. Additionally, our decision to include women with endometriosis who were receiving ovarian suppression to

treat their disease is a strength of the study. While many studies exclude women currently on or having had hormone therapy within the last three months, these treatments are frequently used in women with endometriosis to regulate the menstrual cycle and manage pain. The exclusion of these women does not reflect the clinical reality, in that there are two main cohorts of women with endometriosis; those on ovarian suppression to manage disease and pain, for whom family planning is not a consideration, and those not receiving ovarian suppression, for whom family planning may be a consideration. Furthermore, from the results of our study, the inclusion of women receiving treatment can provide valuable information about biomarker dynamics, and whether or not a biomarker might prove useful in monitoring patient response to treatment.

A third strength of the clinical study was our decision to compare BDNF with other biomarkers of endometriosis. Instead of only describing one novel, putative marker of endometriosis, we chose to describe BDNF as a clinical marker and compare it with two other putative markers (CA-125 and CRP) that had previously been described in the literature. The quantification of circulating BDNF, CA-125, and CRP in the same cohort of women allowed for the direct comparison between clinical markers. Unlike in the systematic reviews of endometriosis biomarkers that attempt to compare studies with varying inclusion criteria and definition of control women, we were able to compare three putative markers in the same women and objectively determine that BDNF is the superior marker for early disease, and the only one that may provide information on patient response to treatment.

Another major strength of our clinical study was our thorough collection of gynecological history, surgical findings, and disease phenotype. We collected this information because recent reviews of endometriosis biomarkers (May et al., 2010; May et al., 2011; Toor et al., 2014) had identified many inconsistencies amongst experimental designs including the failure to account for menstrual cycle stage, stage of disease, duration of disease, location and lesion type, number of lesions, previous treatments, and concomitant disease; the exclusion of women receiving treatment; and the use of varied definitions of control groups (healthy women, women with pelvic pain but no disease, infertile women, etc.). Although much of the information we collected is not detailed in this thesis, we were able to perform sub-analyses on our data (by menstrual cycle stage and stage of disease) in order to assess whether or not our data needed to be stratified by these potential confounders. During our pre-study critical review of the literature several factors shown to affect circulating BDNF concentrations in other studies were identified including: menstrual cycle phase (Begliuomini et al., 2007; Pluchino et al., 2009), oral contraceptive use (Pluchino et al., 2009), post-menopausal age (Lommatzsch et al., 2005; Begliuomini et al., 2007), time of blood collection (Pluchino et al., 2009), melatonin use (Schwertner et al., 2013), patient mass (Lommatzsch et al., 2005), neurological disorders (Karege et al., 2002; Toyooka et al., 2002; Nakazato et al., 2003; Karege et al., 2005; Monteleone et al., 2005; Laske et al., 2006; Ciammola et al., 2007; Laske et al., 2007; Ricci et al., 2010; Scalzo et al., 2010; Rabie et al., 2014; Taurines et al., 2014; Akyol et al., 2015; Piccinni et al., 2015; Polyakova et al., 2015), and cardiovascular disease (Fukushima et al., 2015; Kaess et al., 2015). We

minimized the bias of these factors in our study by: a) performing data sub-analysis by menstrual cycle phase and showing that in our cohort it was not a confounder, b) creating a third experimental group containing women with endometriosis receiving ovarian suppression (oral contraceptives and Lupron), c) recruiting pre-menopausal women, d) collecting blood at the same time of day for all study patients, and e) querying medication use to indirectly assess comorbidities. We were unable to control for patient body mass index as a confounding factor as this information was not collected.

5.7.3: Methodology

The methodology employed in each of the studies is another strength of this thesis. In each chapter the most sensitive assays currently available were employed. Additionally, several complementary methods were used to confirm results obtained using another method. For example in the study presented in Chapter 2, real-time PCR, Western blot, and immunohistochemistry were used to show that BDNF and NTRK2 are present in the uterus. In the same chapter, the antibodies used for immunohistochemistry and Western blots underwent a comprehensive validation to verify their specificity. The antibodies were pre-absorbed using human recombinant proteins prior to immunohistochemistry, and the pre-absorbed antibodies showed reduced immunoreactivity in mouse brain, which was a positive control for BDNF and NTRK2 expression. Recombinant BDNF and NTRK2 were also run on a Western blot, and probed using the tested antibodies, and bands of the appropriate size were visualized. The results of these two tests confirmed that the antibodies

used for immunohistochemistry and Western blots were sensitive and specific, and thus able to detect BDNF and NTRK2.

In the clinical study presented in Chapter 4, the prospective recruitment of women, quantification of circulating BDNF in triplicate, and use of an ELISA with a sensitivity of 15.6pg/mL were among its strengths. The prospective nature of the study allowed for uniform collection of sample and information from the participants, and ensured that all samples were handled and stored in similar conditions. The uniform collection of samples was particulary important for the blood collection, as BDNF concentrations in women have been shown to vary over the day (Pluchino et al., 2009). The prospective nature of our study allowed us to eliminate the daily variation of circulating BDNF as a potential confounder by ensuring that all blood samples were collected at the same time of day (morning) for every woman enrolled in the study. Additionally, when we quantified circulating BDNF we chose to measure in triplicate using a sensitive ELISA to ensure the greatest accuracy in our results. We also randomized plasma samples on the ELISA plates, had several lot numbers of ELISA kits, and conducted the quantification in batches to ensure that samples were randomized over plates and manufacturer lots, and to minimize sample storage time such that the average time in the freezer was no greater than 6 months. Finally, we validated our choice of plasma separator tube and storage conditions in a separate study where the stability of plasma BDNF collected in five different plasma separators and stored at -20 or -80°C for 1 week, 1, 3, and 6 months was assessed (data not shown). Preliminary results support our choice of separator tube over other commercially available tubes, and validate

the stability of plasma BDNF stored at -80°C for at least 6 months; we have an additional aliquot of plasma in which to quantify BDNF after 12 months of storage. Taken together, the experimental designs and methodology employed throughout each chapter of this thesis contribute substantially to its strengths.

5.8: Limitations of the Thesis

While there are many strengths of the thesis presented herein, there are also a few limitations. Although the experiments were all performed *in vivo*, either in experimental animals or on clinical samples collected during surgery, there were several challenges associated with the experiments.

5.8.1: Animal Experiments

Even though we endeavoured to design and conduct all experiments thoroughly and comprehensively, no experiment is perfect. In Chapter 2 we demonstrated BDNF and NTRK2 immunoreactivity in the uterus of six mammalian species, however the Western blots used to determine which isoforms of BDNF and NTRK2 were present in the uterus were only performed in women. This limitation was a result of having used archived paraffin-embedded tissues for immunohistochemistry, and not having access to fresh or frozen tissue homogenates to perform Western blots. However, as the central focus of this thesis was to demonstrate BDNF and NTRK2 expression in uterine cells and provide a link to endometriosis in women, identifying the uterine isoforms of BDNF and NTRK2 in women should suffice. Furthermore, the isolation of uterine BDNF and NTRK2 isoforms

by Western blot in all species is likely unnecessary, considering the degree of conservation we described amongst species.

Another limitation of the animal studies performed in this thesis is that it is difficult to discern if the results can be extrapolated to another species. In Chapter 3 we demonstrate the regulation of BDNF and NGFR in the murine uterus by estradiol, and subsequently postulate in Chapter 5 that excess estradiol in endometriotic lesions might increase BDNF expression in our proposed model of the role of BDNF in the pathophysiology of endometriosis. Although we found conservation of uterine BDNF and NTRK2 expression across six mammalian species, we demonstrated BDNF and NGFR regulation in only one species. Thus, we can only presume that estradiol regulates BDNF and NGFR in endometriotic lesions, in a manner similar to what we described in the murine uterus. However, evidence in favour of this proposal comes from studies in which circulating concentrations of BDNF were positively correlated with estradiol (Begliuomini et al., 2007; Pluchino et al., 2009), and were significantly lower in post-menopausal women (>48 years old) (Begliuomini et al., 2007) and women on oral contraceptives (Pluchino et al., 2009) than in cycling women. Additionally, plasma BDNF concentrations were significantly higher in the secretory phase of the menstrual cycle as compared to the proliferative phase (Begliuomini et al., 2007; Pluchino et al., 2009), and amenorrheic women had significantly lower plasma BDNF concentrations than cycling women (Begliuomini et al., 2007). Further, post-menopausal women receiving hormone replacement therapy had circulating concentrations of BDNF similar to cycling women in the proliferative phase of the

menstrual cycle, which were significantly greater than post-menopausal women who were not receiving replacement therapy (Begliuomini et al., 2007). Taken together, these reports provide strong evidence supporting the regulation of BDNF by estradiol in women, and suggest the upregulation of BDNF and NGFR in human endometrial and endometriotic cells may be driven by estradiol, as we observed in the murine uterus. However, future experiments should strive to demonstrate estradiol regulation of BDNF and NGFR in primary endometrial cells, and/or human endometrial cells lines. Additionally, it would be useful to determine if BDNF and its receptors are expressed in each type of endometriotic lesion. Each of these experiments would help to further substantiate our proposed model of the role of BDNF in the pathophysiology of endometriosis.

5.8.2: Clinical Experiment

The clinical study presented in Chapter 4 described increased circulating concentrations of BDNF in women with endometriosis as compared to those without. However, one of the limitations of our clinical experiment is our relatively small number of women with Stage I/II disease. Nevertheless, we were not prevented from attaining significance in our statistical comparison between women with Stage I/II disease (N=12) and controls (N=36). Our limited access to Stage I/II endometriosis is because McMaster University Medical Centre is a tertiary care centre for endometriosis. As such, patients are referred for minimally invasive surgery by primary or secondary care providers, generally after having seen various specialists, undergoing several years of treatment for pelvic pain, and having many rounds of medical imaging. It is therefore not overly surprising that the length of time

between a patient presenting with symptoms of endometriosis until confirmed diagnosis is 11.7 years in the U.S. (Ballard et al., 2006). Unfortunately, because women do not see a surgeon until several years after the onset of pelvic pain, their disease has often progressed past Stage I/II, and thus the recruitment of women in these stages is challenging.

Similarly, the recruitment of women with pelvic pain but without endometriosis (symptomatic controls) was also challenging because many women suspected to be controls prior to surgery were diagnosed with endometriosis during surgery. With a limited pool of symptomatic controls, we decided to circumvent this issue by recruiting healthy, asymptomatic women to increase the number of controls in the clinical study. However, this was not without limitation because our asymptomatic women did not undergo surgery to confirm the absence of endometriosis. It is possible for a woman to be unaware she has endometriosis, and thus it is possible that one or several of our asymptomatic controls had endometriosis that was not clinically manifest. Fortunately, if any of the asymptomatic controls recruited into the clinical study did have endometriosis, it would bias our results towards the null hypothesis that no difference in circulating BDNF exists between women with and without endometriosis. For this reason we are confident in including asymptomatic women in our study.

Another limitation of the clinical study was our exclusion of three control women with adenomyosis. Any woman with adenomyosis was excluded from the study due to the potential for confounding, because women with adenomyosis also have excess endometrial

tissue. Thus, their inclusion was likely to confound results and bias them towards the null hypothesis. Therefore, we identified an additional factor that likely affects circulating concentrations of BDNF, adenomyosis. We suggest that in the future, women with adenomyosis should be included in an assessment of plasma BDNF as a biomarker of endometriosis, but as separate cohort to better understand the relationship between this condition and circulating BDNF.

A final limitation of the clinical study was that it was performed at one centre, and thus requires external validation. Although the results of our study are promising, there is a need for a careful, rigorous, large scale, and collaborative assessment of BDNF as a clinical marker of endometriosis. It is only after external validation, replication, confirmation of our results by other research groups, and further study that BDNF might be adopted clinically as a means of strongly suspecting Stage I and II disease, and monitoring progression and patient response to treatment. This is one of our future directions.

5.9: Future Directions

This thesis presents evidence supporting a theory linking enhanced estrogen-regulated expression of BDNF with the pathophysiology of endometriosis. While the work presented here provides an excellent foundation for future experiments, several research questions remain unanswered. As this is the first comprehensive description of BDNF and its receptors in the mammalian uterus, there are innumerable opportunities for future research.

First and foremost, the function of BDNF and its receptors in the uterus under both physiological and pathological conditions is mainly unknown. Therefore, future directions should include an assessment of BDNF function *in vivo* and *in vitro*, in the uterus using an animal model and cultured uterine cells. The use of knockout animals can often provide functional information about a protein of interest. Unfortunately because BDNF is central to proper neuronal development and function, BDNF null mice are not viable and die shortly after birth (Ernfors et al., 1994; Jones et al., 1994; Conover et al., 1995). The generation of a conditional deletion of the BDNF gene in cells expressing the progesterone receptor (uterus, ovary, oviduct, pituitary, and mammary glands) can likely be achieved by crossing floxed BDNF mice with progesterone receptor Cre knockin mice originally generated by Soyal et al., 2005. Upon generation of conditional knockout mice, the uterine function of BDNF can begin to be elucidated. A direct comparison between knockouts and wild type mice would help clarify whether or not BDNF is involved in uterine development, physiology, and reproduction. Specifically, the pathways activated by BDNF in the brain and nervous system (proliferation, adhesion, angiogenesis, resistance to apoptosis) should be contrasted between knockouts and wild type mice. To complement and expand upon these experiments, endometrial epithelial and stromal cell lines can be cultured in the presence or absence of recombinant BDNF, and the effect on proliferation, adhesion, angiogenesis, and apoptosis can be determined using commercially available assays. Alternately, primary endometrial cells obtained by pipelle biopsy can be quantified, equally divided and treated with BDNF or vehicle, and the aforementioned pathways can be assessed as above. Another option would be to knock down BDNF translation in culture

using small interfering RNAs (siRNAs) and comparing activated pathways with cells transfected with scrambled siRNA. Thus, there are plenty of options to explore the function of BDNF and its receptors in the mammalian uterus, and in uterine cell types.

Another set of experiments aimed at expanding upon the clinical data presented in Chapter 4 of this thesis is warranted. Based on the results of our study, we speculate that a larger scale study with an increased ability to stratify patients into groups will identify additional factors (burden of disease, lesion type, lesion location, medication use, comorbidities) that affect circulating concentrations of BDNF, which should be controlled for when employing BDNF as a clinical marker of endometriosis. We have established that there is a relationship between circulating BDNF and endometriosis, however there are still many unanswered questions surrounding their association. First, to provide external validation to the results of our study, it should be replicated by an independent research group, preferably in another geographical location, with greater access to women with early stage disease. Secondly, another, larger scale study should be designed and conducted. In the study, any women undergoing laparoscopy should be prospectively recruited into the large-scale, multi-site study, and given a questionnaire including but not limited to date of last menstrual period, gynecological history, medication use, concomitant disease, pelvic pain, duration of disease, and socio-demographics. Prior to surgery, blood should be collected and stored as per our study protocol. During surgery, the presence or absence, number and type of lesions, disease burden, and diagram of lesion location need to be rigorously documented, and pathological confirmation of disease should be obtained. Once this information has

been collected, women can be categorized into cases, those with endometriosis, and controls, those with other conditions. They should be further sub-categorized by stage of endometriosis, menstrual cycle phase, and medication use. Ideally, there would be the possibility of following the recruited women and quantifying circulating BDNF at three, six, and nine months post-surgery to determine if increasing BDNF can provide an early indicator of pain re-emergence. Further, the effect of specific medications, and menstrual cycle phases on circulating BDNF can be determined. Essentially, this study should aim to increase the size of the study population, optimize the timing of BDNF quantification by assessing whether quantification during one cycle phase over the other provides a better indicator of disease in a larger cohort, establish a temporal relationship between circulating BDNF, reoccurrence of pain and disease, confirm the association between elevated BDNF and Stage I/II disease and lesion activity, and further explore the effect of specific endometriosis treatments on circulating BDNF. Additionally, the inclusion of BDNF in a panel of putative endometriosis biomarkers should strongly be considered. We also suggest that a large scale, rigorously controlled comparison between circulating concentrations of BDNF in women recruited in the proliferative phase (day 6-8), with Stage I and II disease, compared with symptomatic controls recruited in the proliferative phase (day 6-8) using the methodology described in Chapter 4 offers the opportunity to further substantiate BDNF as a useful clinical marker of early stage endometriosis. Based on our study, and that of Giannini et al., 2010, we speculate that the recruitment during the proliferative phase, and focus on Stage I and II disease are likely to increase the separation between the mean circulating concentration of BDNF in cases and controls.

Finally, although the presence of NGF in endometriotic lesions has been documented (Anaf et al., 2002; Mechsner et al., 2007), demonstration of the expression of BDNF and its receptors in endometriotic lesions is lacking. Considering that we found a significant difference in circulating BDNF, but not NGF in women with endometriosis when compared to controls, we suggest that isolating BDNF and its receptors in the endometriotic lesions would be useful. Showing that BDNF and its receptors are present in the endometriotic lesions, particularly active lesions will add integrity to our model outlining the postulated role of BDNF in the pathophysiology of endometriosis. Further, after determining that BDNF is expressed by the endometriotic cells, antagonizing the BDNF-NTRK2 interaction would offer a novel therapeutic avenue for endometriosis treatment. Most importantly, if appropriately targeted, suppressing BDNF in the endometriotic lesions might be the first treatment for endometriosis that does not suppress fertility as a side effect.

5.10: Summary and Importance

Taken together, the data presented in this Ph.D. thesis advances our limited knowledge of uterine neurotrophins, and establishes a link between circulating BDNF concentration and endometriosis. Our data expand the literature by resolving the equivocal nature of BDNF and NTRK2 expression in the uterus. We demonstrated BDNF and NTRK2 in the uterus of six mammalian species, including two that menstruate and four that do not. To build upon and complement our results, we highlighted the positive uterine regulation of BDNF and its low affinity receptor NGFR by estradiol. Even though there were relatively few

studies suggesting an association between BDNF and endometriosis, we conceived a model of its role in disease pathophysiology. Encouraged by results of our animal studies, and knowing that the endometriotic lesions are exposed to excess estradiol in women with endometriosis, we assessed circulating BDNF for its ability to differentiate between women with and without endometriosis. We discovered that the plasma concentration of BDNF is greater in women with endometriosis, particularly in those with Stage I and II disease, compared to controls. We also found that circulating concentrations of BDNF were lower in women with endometriosis who were treated with ovarian suppression compared to women with endometriosis who were untreated. Therefore, these data suggest that BDNF might provide an opportunity to monitor patient response to endometriosis treatment. When we compared BDNF with other putative biomarkers of endometriosis, CA-125 and CRP, we found BDNF to be superior.

Endometriosis is a condition that is under-diagnosed as its symptoms mimic other gynecological and gastrointestinal disorders, and clinical biomarkers do not exist (May et al., 2010; Giudice, 2010). Currently, the only definitive diagnosis occurs after laparoscopic visualization of endometriotic lesions, and subsequent histological confirmation of disease. However, even after laparoscopic surgery, the diagnosis may be delayed because small lesions are not easily viewed during surgery (Giudice, 2010) and some lesions are misclassified at pathology (Wanyonyi et al., 2011). In most cases, the length of time between a patient presenting with symptoms of disease until confirmed diagnosis is prolonged. In the US, this delay averages 11.7 years (Ballard et al., 2006). This is

problematic as the disease generally worsens over time, and its chronic nature is a burden on the healthcare system. Endometriosis is one of the largest national healthcare expenditures (Gao et al., 2006; Simoens et al., 2007; Simoens et al., 2012) with the annual cost being approximately $69.4 billion in the U.S. (Simoens et al., 2012; reviewed in Burney and Giudice, 2012), and $1.8 billion in Canada (Levy et al., 2011) in 2009. This is significantly more than comparable chronic conditions (Simoens et al., 2007). Here we provide evidence for the involvement of BDNF in the pathophysiology of endometriosis by demonstrating expression of BDNF and all of its receptors in the uterus, highlighting its regulation in uterine cells by estradiol, and confirming that circulating concentrations of BDNF are elevated in women with endometriosis compared with controls. Thus, we strongly recommend BDNF as a candidate clinical marker of endometriosis, and encourage further research aimed at determining its role in the pathophysiology of endometriosis, and exploring its therapeutic potential.

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Appendix I: Supplemental Figures

Supplemental Information for Paper 2 (Chapter 3)

Total N values remaining for statistical comparisons, after removal of outliers and technical errors. Non-detectable values were assigned a random number between the limit of detection for the gene or protein of interest and zero using the random number generator in the SigmaStat software package.

BDNF mRNA Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	3
Outlier	0	1	1	1
Error	0	0	0	0
Total N	8	17	8	14

NTRK2 mRNA Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	2	2	0
Outlier	1	1	1	1
Error	0	0	0	0
Total N	**7**	**17**	**8**	**14**

NGFR mRNA Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	0	0	0	0
Error	0	0	0	0
Total N	**8**	**18**	**9**	**15**

Supplementary Figure S1 Total *N* values for all comparisons. A list of the number of non-detectable observations, outliers, and omissions in PCR and western blot data. Briefly, non-detects were assigned a computer-generated random number between the limit of detection for that particular gene or protein and zero. Outliers and technical errors were omitted from analysis. Statistical outliers in the data were determined using the Dixon's *Q* test or the Grubb's test.

SORT1 mRNA Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	0	0	0	0
Error	0	0	0	0
Total N	**8**	**18**	**9**	**15**

BDNF mRNA Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	1	2	0	0	0
Outlier	0	0	0	1	0
Error	0	0	0	0	0
Total N	**5**	**6**	**6**	**5**	**6**

NTRK2 mRNA Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	3	1	2	0
Outlier	0	1	1	0	1
Error	0	0	0	0	0
Total N	**5**	**5**	**5**	**6**	**5**

Supplementary Figure S1 Continued

NGFR mRNA Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	1	2	1	2	1
Outlier	0	0	0	0	0
Error	0	0	0	0	0
Total N	**5**	**6**	**6**	**6**	**6**

SORT1 mRNA Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	1	2	1	0	0
Outlier	1	0	0	0	0
Error	0	0	0	0	0
Total N	**4**	**6**	**6**	**6**	**6**

BDNF 25kDa Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	0	1	0	0
Error	0	0	0	0
Total N	8	11	9	10

Supplementary Figure S1 Continued

BDNF 27kDa Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	1	1	0	0
Error	0	0	0	0
Total N	7	11	9	10

BDNF 37kDa Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	1	0	0
Outlier	0	1	0	0
Error	0	0	0	0
Total N	8	11	9	10

BDNF 40kDa Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	0	0	0	1
Error	0	0	0	0
Total N	8	12	9	9

Supplementary Figure S1 Continued

NTRK2 Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	1	0	0	0
Error	0	0	0	0
Total N	7	12	9	10

NGFR Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	1	0	1	1
Error	0	0	0	0
Total N	7	12	8	9

SORT1 Protein Quantification in Cycling Mice

	Proestrus	Estrus	Metestrus	Diestrus
Non-detectable	0	0	0	0
Outlier	0	0	0	0
Error	0	0	0	0
Total N	8	12	9	10

Supplementary Figure S1 Continued

BDNF 25kDa Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	0	0	0	0
Outlier	0	0	0	0	0
Error	0	0	0	0	0
Total N	**5**	**6**	**6**	**6**	**6**

BDNF 27kDa Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	0	0	0	0
Outlier	0	0	0	1	0
Error	1	0	0	0	0
Total N	**4**	**6**	**6**	**5**	**6**

BDNF 37kDa Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	1	0	0	0
Outlier	0	0	0	0	0
Error	1	0	0	0	0
Total N	**4**	**6**	**6**	**6**	**6**

Supplementary Figure S1 Continued

BDNF 40kDa Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	0	0	0	0
Outlier	0	0	0	0	0
Error	1	1	0	0	0
Total N	**4**	**5**	**6**	**6**	**6**

NTRK2 Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	0	0	0	0
Outlier	0	1	0	0	0
Error	0	0	0	0	0
Total N	**5**	**5**	**6**	**6**	**6**

NGFR Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	2	0	1	0
Outlier	0	0	0	1	0
Error	1	0	0	0	0
Total N	**4**	**6**	**6**	**5**	**6**

SORT1 Protein Quantification in Hormone Replacement Mice

	OVX	Saline	Estradiol	Progesterone	Estradiol+Progesterone
Non-detectable	0	0	0	0	0
Outlier	0	0	0	0	0
Error	0	0	0	0	0
Total N	**5**	**6**	**6**	**6**	**6**

Supplementary Figure S1 Continued

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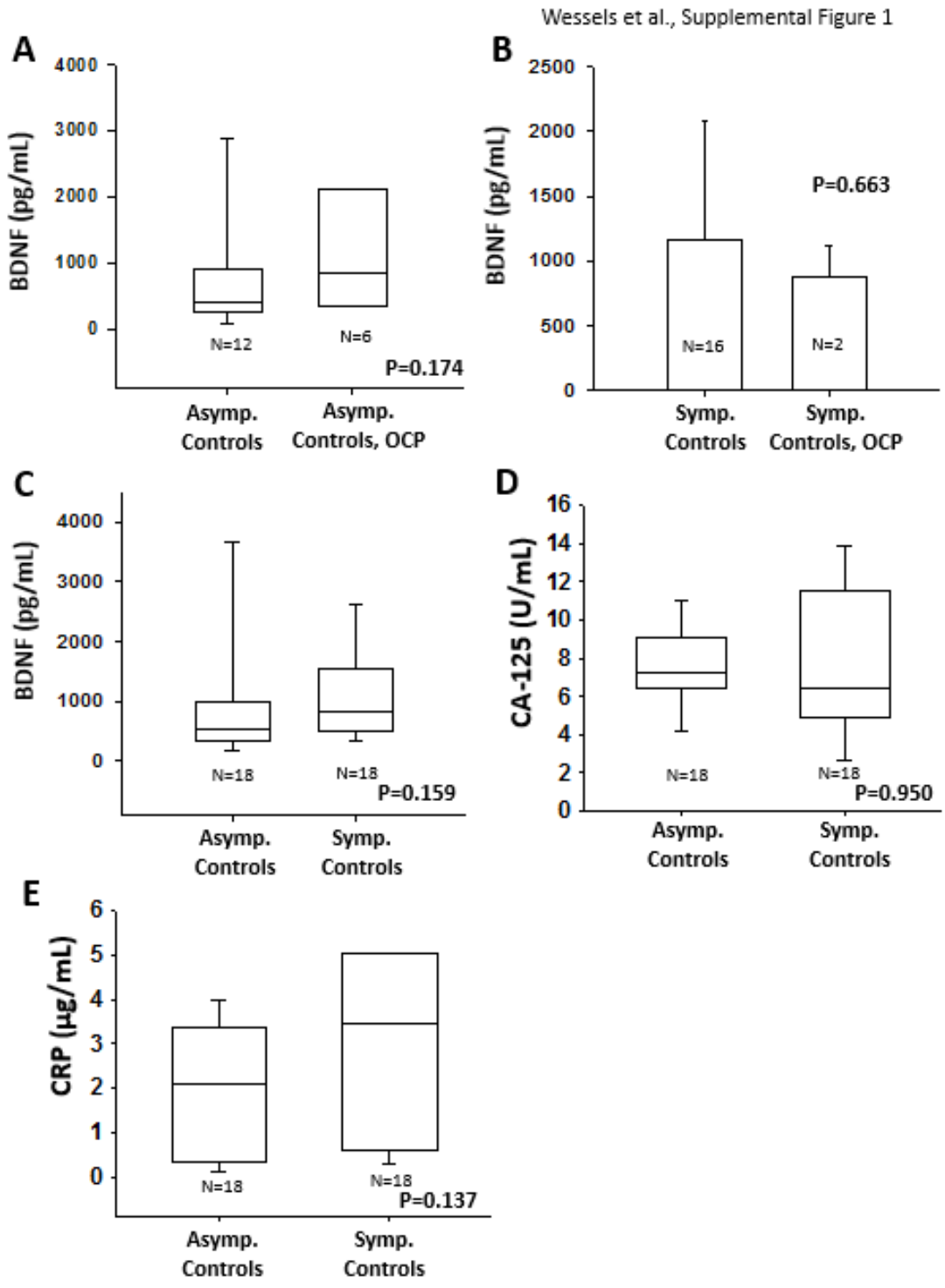

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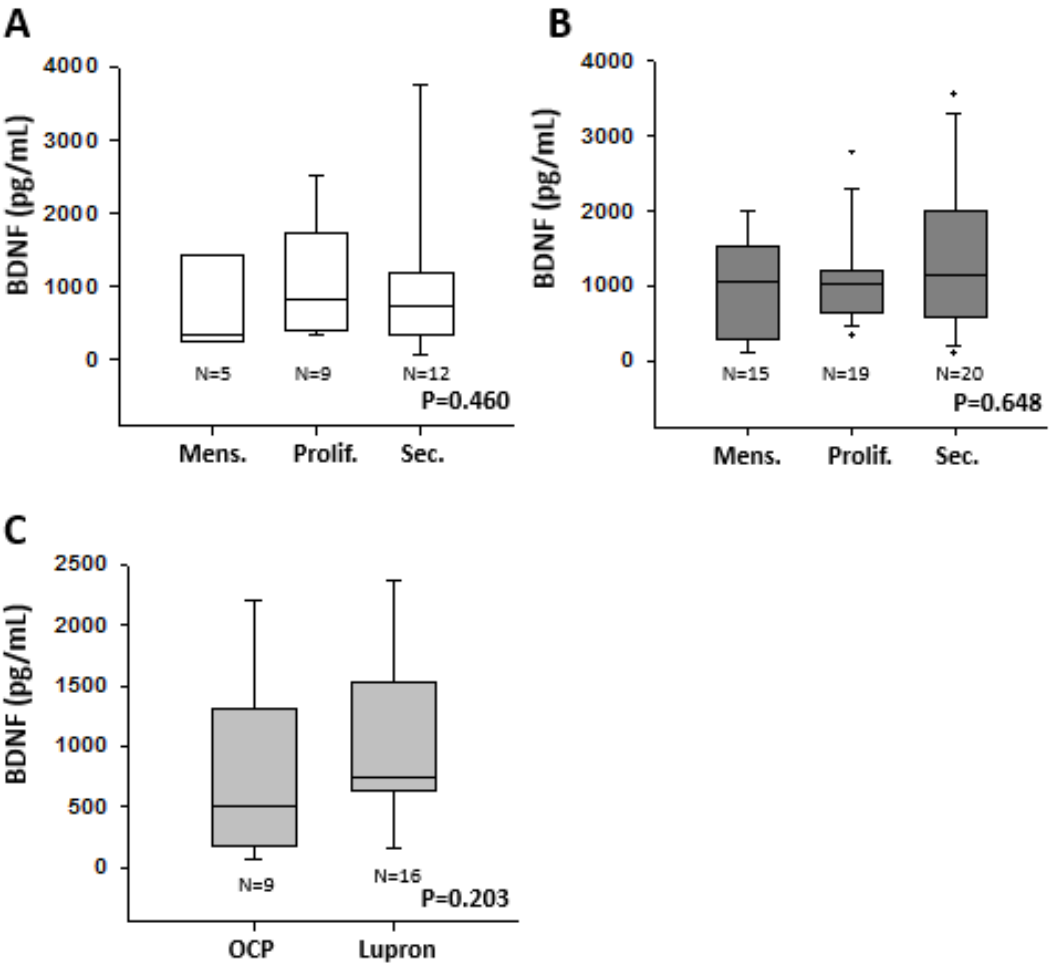

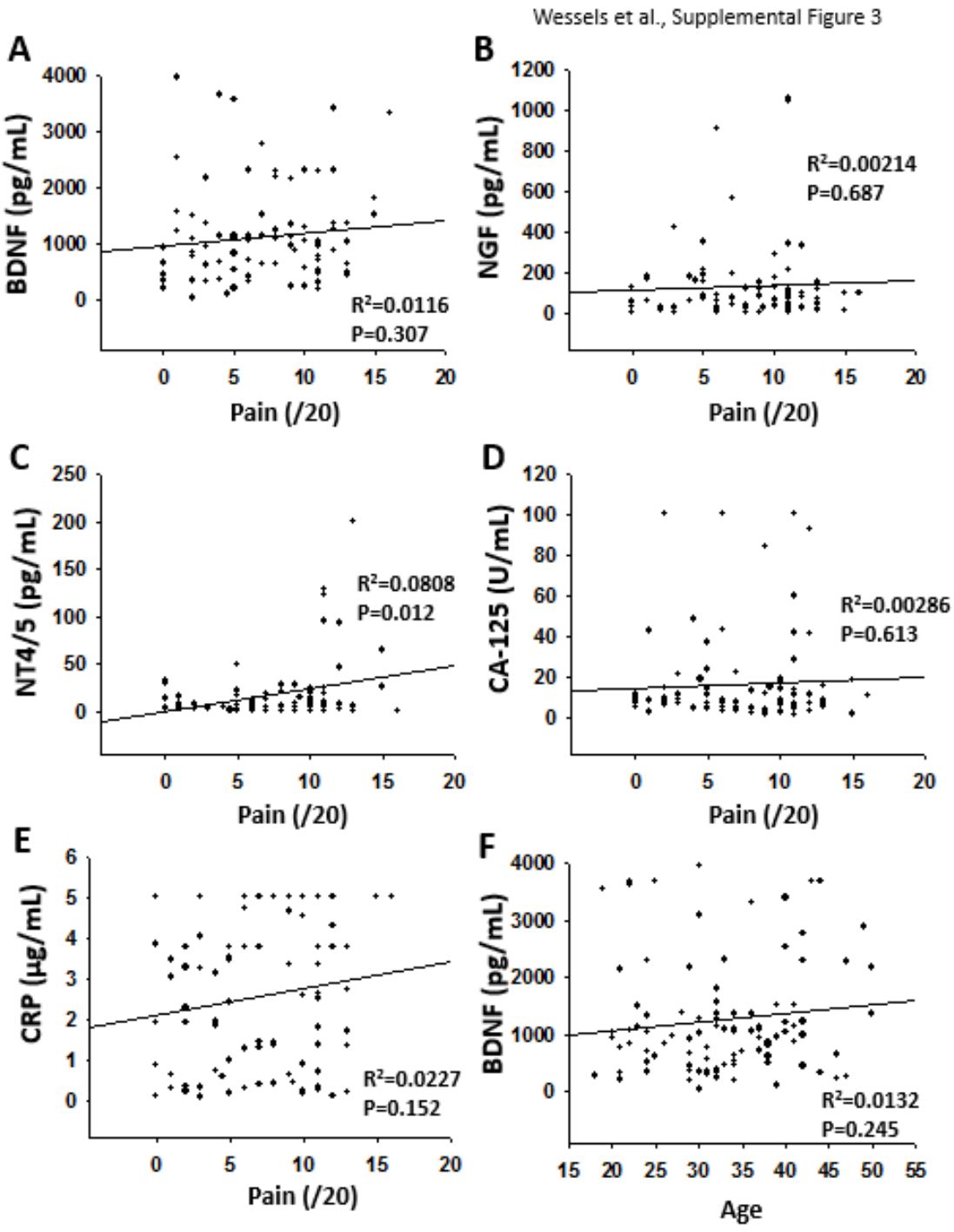
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A
BDNF (pg/mL)
R²=0.0116
P=0.307
Pain (/20)
B
NGF (pg/mL)
R²=0.00214
P=0.687
Pain (/20)
C
NT4/5 (pg/mL)
R²=0.0808
P=0.012
Pain (/20)
D
CA-125 (U/mL)
R²=0.00286
P=0.613
Pain (/20)
E
CRP (µg/mL)
R²=0.0227
P=0.152
Pain (/20)
F
BDNF (pg/mL)
R²=0.0132
P=0.245
Age

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Chapter 2: The Brain-Uterus Connection: Brain Derived Neurotrophic Factor (BDNF) and Its Receptor (Ntrk2) Are Conserved in the Mammalian Uterus
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Chapter 3: Estrogen Induced Changes in Uterine Brain-derived Neurotrophic Factor and Its Receptors

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Figure 5: Interaction between Inflammation, Neurotransmitters and Pain in Ectopic Endometriotic Lesions.

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Figure 7: Proposed Role of Brain-derived Neurotrophic Factor (BDNF) in the Pathophysiology of Endometriosis.

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