DIFFERENTIAL MICRORNA EXPRESSION IN ENDOMETRIOTIC IMPLANTS

ASSESSING DIFFERENTIAL MICRORNA EXPRESSION IN ENDOMETRIOTIC IMPLANTS

By:

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2017) Hamilton, Ontario (Medical Sciences)

TITLE: Assessing differential microRNA expression in endometriotic implants.

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NUMBER OF PAGES: xvii, 101

ABSTRACT

Endometriosis is an estrogen-dependent disease that is characterized by the growth of endometrial tissue outside of the uterine cavity. The most common endometriotic lesions are ovarian endometrioma, peritoneal lesions, and deeply-infiltrating endometriosis. Ten percent of women in reproductive age are affected, a gross underestimate due to the delay in diagnosis and non-specific symptoms. The etiology of endometriosis is not well understood, making diagnosis difficult, and treatments suboptimal. Currently, laparoscopic surgery is the gold standard for diagnosis, however this method is invasive, costly, and physicians are often reluctant to send their patients to surgery without certainty of disease. It is therefore a research priority to identify a minimally-invasive biomarker for endometriosis.

Over the years, the search for a biomarker has shifted from a single circulating biomarker, to a panel of circulating biomarkers, and finally to the advent of newer technologies. The studies of proteomics, genomics, phenomics, and metabolomics have shown some promise thus far. MicroRNAs, a discovery of genomics, are short, non-coding RNA strands that regulate mRNA expression by silencing or degrading the transcript. The dysregulation of miRNAs have been shown to contribute to the pathology of many gynecological conditions, and have shown to be dysregulated in endometriosis. To date however, results have been underwhelming due to differences in methodologies and failure to consider endometriosis as a heterogeneous disease. Three miRNAs were studied based on their prevalence in the literature (miR-9, -21, and -424), and three others (miR-10a, -10b, and -204) were measured based on their association with BDNF. In the

current study, miR-204 expression was significantly lower (p=0.0016) in the eutopic endometrium of women with endometriosis compared to controls. Relative expression of miR-21, miR-424, and miR-10b differed significantly ($p<0.05$) across lesion types in women with exclusively endometriomas, peritoneal or deep-infiltrating lesions. Corresponding BDNF expression in the lesion types were inversely correlated to miRNA expression suggesting these miRNA regulate BDNF and are implicated in endometriosis pathology. Due to the findings that miRNAs are differentially expressed between endometriotic lesions, this study also suggests that, different lesion types are biochemically distinct.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. Warren Foster. Not only did he take me into his lab 6 months into my degree, but did so graciously and provided me with an enriched environment to develop my skills as a researcher. I am thankful for all of the personal and academic advice I have received during the course of my degree. Furthermore, Dr. Foster has given me the opportunity to travel and present my research, helping me become more confident communicating my research, and having the chance to meeting world-renowned leaders in the field. I do not think I could have come across another supervisor who is as supportive and selfless as Dr. Foster.

I also would like to thank the lab mates I have had over the past couple of years: Dr. Hayley Furlong, Dr. Jocelyn Wessels, Mike Tsoulis, Allegra Drumm, and Nicholas Stalteri, who have helped me along at different times of my degree. I'm grateful that Hayley took the time to orient me with the lab, introduced me to many people at conferences, showed me how to get my project started, and taught me the basics of RT-qPCR. Jocelyn has helped me throughout my degree, and has always been available when I had questions about previous experiments; she was always willing to dedicate some time to help me. I'd like to thank Mike for teaching me how to do a western blot and for giving me general advice that has proven useful in my studies. Allegra was the first to show me the OR and taught me how to process the biological samples I would eventually be using for the study. Finally, I'd like to thank Nicholas, a very bright undergraduate student with an aptitude for numbers, who helped me understand my statistical analyses when I first started.

I would like to thank my committee members, Dr. Nicholas Leyland and Dr. Gurmit Singh. I appreciate the time they have both taken to help me with my Master's, and for their guidance and feedback during my committee meetings.

Furthermore, I would like to thank Nicholas Leyland once again, for allowing me to observe surgeries to obtain samples for the study. I value the fact that he took time to step away from surgery to help me understand the process. I would like to thank Annette Bullen as well, who has helped with recruitment for the study and who was always willing to help me when I needed it. She so kindly sat down with me to discuss my future plans of becoming a nurse, and I was very grateful to have been able to speak with her about that.

I would also like to take a moment to thank all of the study participants who so gratefully provided samples for the study in hopes of progressing endometriosis research. Without their generosity the research would not be possible.

Finally, I would like to thank my family and friends, specifically my partner Gael, who has shown me so much support and who has been the voice of reason during my times of struggle throughout my degree. I could not have asked for a greater group of people to help me through my Master's degree. With them, I would not have the knowledge or the confidence I do today.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
β	beta
Δ	delta
γ	gamma
κ	kappa
AGO	Argonaute
ANOVA	Analysis of variance
Bax	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMI	Body mass index
CA-125	Cancer antigen-125
DIE	Deep-infiltrating endometriosis
DNA	Deoxyribonucleic acid
ECL	Electrochemiluminescence
EEC	Endometrioid endometrial cancer
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH-a	Gonadotropin-releasing hormone agonist
HGF	Hepatocyte growth factor
HIF1-α	Hypoxia-inducible factor 1-α
IFN-γ	Interferon-γ
IL-6	Interkeukin-6
LF-PVDF	Polyvinylidene difluoride

LNG-IUD	Levonorgestrel intrauterine device
miRNA/miR	microRNA
MMPs	Matrix metalloproteinases
mRNA	messenger RNA
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NGS	Next generation sequencing
NK cell	Natural killer cell
NSAIDs	Non-steroidal anti-inflammatory drugs
NT-4/5	Neurotrophin-4/5
Ntrk2	Neurotrophic receptor tyrosine kinase 2
OCPs	Oral contraceptive pills
OMA	Ovarian endometrioma
PE	Peritoneal lesion
piRNA	Piwi-interacting RNA
Pol III	Polymerase III
PTEN	Phosphatase and tensin homolog
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
rASRM	revised American Society for Reproductive Medicine
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction

SD	Standard deviation
SDC-1	Syndecan 1
sICAM-I	soluble Intracellular Adhesion Molecule I
siRNA	Small-interfering RNA
SN	Sensitivity
SNORD	small nucleolar RNAs
SNP	single-nucleotide polymorphism
SP	specificity
TBST	Tris-buffered saline – Tween 20
TNF-α	Tumour necrosis factor α
TSP-1	Thrombospondin 1
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

DECLARATION OF ACADEMIC ACHEIEVEMENT

All of the experiments were performed by me, except for the histological staining to determine menstrual cycle stage in eutopic endometrium, which was performed by Mike Tsoulis and Jocelyn Wessels.

CHAPTER 1: INTRODUCTION

1.1 What is Endometriosis?

Endometriosis is an estrogen-dependent disease of unknown etiology, characterized by the growth of endometrial tissue in ectopic locations. Endometriosis affects 6-10% of women of reproductive age.[1,2] This is likely an underestimate of the true prevalence, due to diagnostic delay, and comorbidities that present with similar syptoms.[3–5] The majority of endometriotic lesions are localized in the pelvic peritoneum, ovaries, and rectovaginal septum, leading to clinical symptoms such as chronic pelvic pain, dysmenorrhea, excessive bleeding, and in some cases infertility.[6,7,8] It is the third leading cause of gynecological hospitalizations in the United States, as well as the leading cause of hysterectomies.[2,9] Based on a multi-centre study across ten countries, it was determined that affected women lost an average of 10.8 hours of work each week, due to reduced efficiency at work.[3] In 2009 alone, the total annual societal costs were estimated at $69.4 billion in the United States, imposing a heavy burden on women and the healthcare system.[10]

The longest standing theory for the implantation of endometriotic lesions is retrograde menstruation, presented by Sampson in 1927[11] (***Fig. 1)***. Although widely accepted, Sampson's theory fails to account for the wide disparity between the 76-90% of women who experience retrograde menstruation and the reported 10% prevalence of endometriosis.[12–14] While this may seem like an important counterargument, many other groups have proposed theories that work in conjunction with Sampson's theory, involving stem cells, oxidative stress, inflammation and hormones.[6,14–23] Therefore, it is suggested

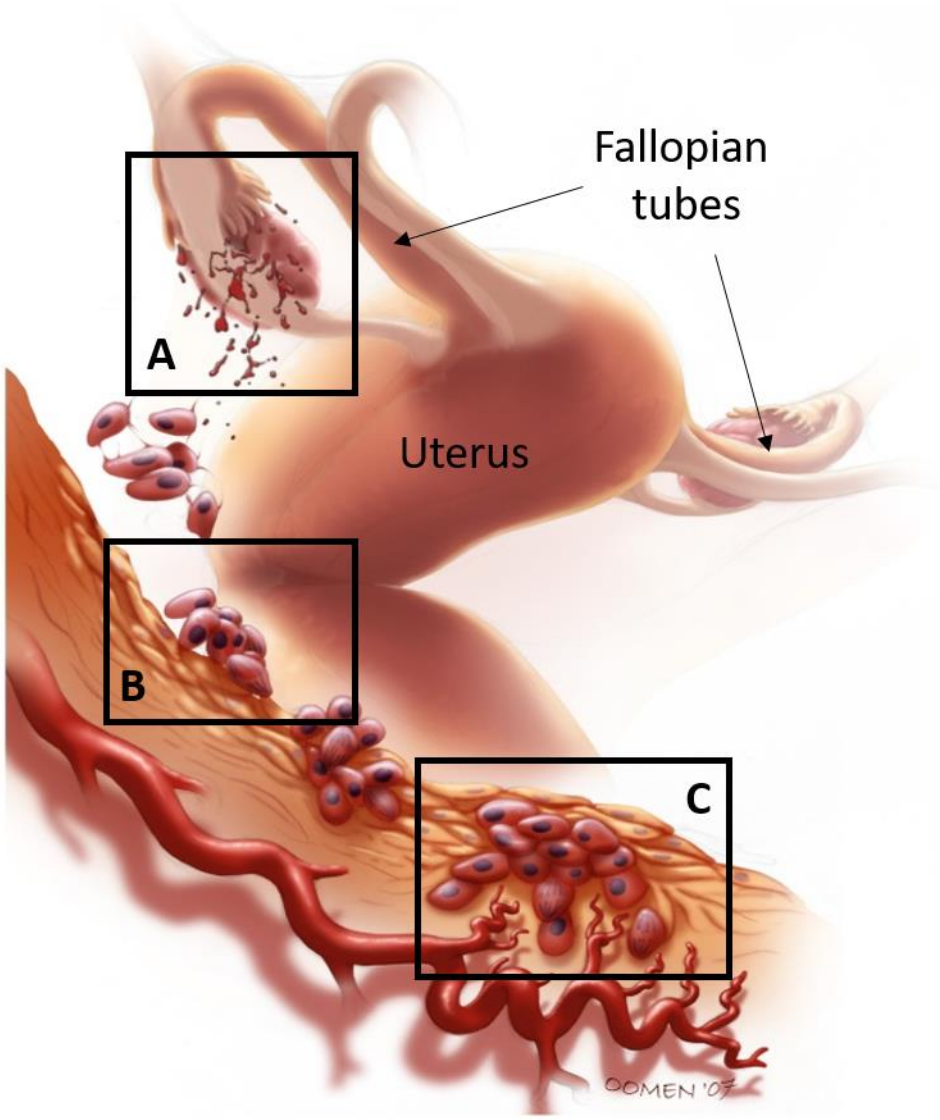


Figure 1. *Retrograde Menstruation. Sampson's theory of retrograde menstruation suggests that endometriosis manifests after the backflow of menstrual debris, via the fallopian tubes, which allows itself to implant onto the peritoneum. (A) Ovarian endometrioma, (B) established peritoneal lesions, and (C) deeply-infiltrating endometriosis are the three most common manifestations of disease.*

that the pathogenesis of endometriosis is multifactorial involving dysregulation of multiple biological pathways.[6,9,24] Another popular theory involves extrauterine cells that differentiate into endometrial cells, a process called coelomic metaplasia.[14] This theory states that endometriosis develops from the metaplasia of specialized cells in the mesothelial lining of the visceral and abdominal peritoneum, influenced by hormonal and immunological factors.[14] Although numerous biochemical differences between women with endometriosis and healthy controls have been documented, none have been found to have adequate sensitivity and specificity to serve as clinical tools for the diagnosis of this enigmatic disease.[25–27] Hence, identification of clinical markers of endometriosis remains a high priority issue that has yet to be resolved. A novel biomarker would ideally provide insight for the cause the disease, and can provide a good framework upon which new treatments can be developed. Treatments for endometriosis currently remain sub-optimal.

1.2 Current Treatments

To date, there is no cure for endometriosis, and the current treatment options are not desirable for many affected women. While most treatments reduce pain associated with endometriosis, they do so by suppressing ovarian function, and ultimately repressing fertility (**Table I**).

Current first-line treatment for symptoms of chronic pelvic pain include but are not limited to: non-steroidal anti-inflammatory drugs (NSAIDs), combined oral contraceptive pill (OCPs), traditional progestins such as norehisterone, and newer progestins such as dienogest.[9,28–31] Progestins limit the growth of the endometriotic

Table I. Proposed treatment options for endometriosis-related pain.

Medication	Common market names	Type of therapy	Suppresses ovulation?	Potential Side Effects	Notes
NSAIDs[32]	Advil, Motrin	First-line	No	Nausea, vomiting, bloating, dizziness, acid reflux	
Cyclic OCPs[32]	Alesse, Ortho Tri-cyclen	First-line	Yes	Nausea, weight gain, breast tenderness, dizziness, depression	Continuous OCPs are second-line therapies
Progestins[30, 33,34]	Oral: Dienogest, Visanne Vaginal: LNG-IUD	Second and third-line	Yes	Nausea, weight gain, breast tenderness, depression, fluid retention, amenorrhea	LNG-IUD can be changed every 5 years
Androgens[35 ,36]	Danazol	Second or third-line	Yes	Androgenic side effects (deepening of voice, hair growth, acne), hot flushes	Not commonly used
Aromatase inhibitors[36]	Letrozole	Third-line	Yes	Bone fracture, edema, fatigue, dizziness	
GnRH agonists[32,37]	Lupron (subcutaneous depot)	Second or third-line	Yes	Hypoestrogenic side effects (bone mineral density loss, vasomotor symptoms, decreased libido)	Hormonal add back therapy with estrogen-progestin can prevent bone loss
GnRH antagonist[38]	Elagolix	Second or third-line	Yes	Hot flushes, headache, nausea	Oral: can adjust dose to limit vasomotor symptoms and bone loss

NSAIDs= non-steroidal anti-inflammatory drugs; OCPs= oral contraceptive pills; LNG-IUD = levenorgestrel intrauterine device; GnRH= gonadotropin-releasing hormone

lesions by inducing decidualization followed by atrophy, and decreased inflammatory markers in the peritoneum.[30] Furthermore, dienogest imposes very little androgenic, and estrogenic influence on metabolic activity, but side effects of weight gain, increased blood pressure, and nausea are common.[30,34,39] It promotes both an anovulatory and antiproliferative effect, while blocking the secretion of cytokines in the endometrium.[30,33]

Second-line treatments are used if first-line medical therapies are unsuccessful or contraindicated. Second-line therapies can include gonadotropin-releasing hormone (GnRH) agonists, levanorgestrel intrauterine devices (LNG-IUDs), and opioid analgesics.[9] GnRH agonists exert therapeutic effects by binding to the GnRH receptor, depleting the pituitary of endogenous gonadotropins.[40] The hypoestrogenic state disrupts the menstrual cycle, promoting endometrial atrophy and amenorrhea.[40] Although the hypoestrogenic side effects such as decreased libido and bone mineral density loss can be a major drawback, this can be partially reversed with hormonal add-back therapy, typically progestins with or without a low dose of estrogen. Add-back therapy has improved compliance, duration of therapy, and limits side-effects such as bone loss.[37] Intrauterine devices (IUD) such as the levenorgestrel-IUD have been shown to improve chronic pelvic pain, dysmenorrhea, and dyspareunia, as well as reducing the size of endometriotic lesions, without producing hypoestrogenic side effects.[41,42] For women without a desire to conceive this is advantageous, especially because medical intervention only occurs twice, upon insertion of the device and its removal (typically 5 years).

Recently, GnRH antagonists have become a newer and improved method of managing pain. These non-peptides that can be taken orally, exert their effect via direct

gonadotropin suppression which inhibits endometriotic cell proliferation and invasion.[36,38] GnRH antagonists are more resistant to degradation by peptidases compared to the native GnRH, due to an amino acid substitution resulting in a longer receptor occupancy and a longer half-life.[30] It is a dose-dependent method of treatment that is optimized when efficient pain relief is achieved while maintaining sufficient estradiol levels to avoid vaginal atrophy, bone density loss, and hot flushes[36]. In a recent clinical study on GnRH-antagonist elagolix, consisting of two similar double-blind, randomized, phase 3 trials, the percentage of women with an improvement in dysmenorrhea was 46.4% in a lower dose group (150mg daily) and 75.8% in a higher dose group (200 mg twice daily), compared to 19.6% in a placebo group.[38] The second trial showed similar results with 43.4%, 72.4%, and 22.7% showing a clinical response to dysmenorrhea, respectively.[38] Another advantage to oral GnRH antagonists is that hypoestrogenic effects can be reversed much quicker after cessation of medication, unlike GnRH agonists whose subcutaneous depot injections can last for three months.

Unfortunately, for women trying to conceive, the majority of medical therapies for pain are contraceptive in nature. The only way to potentially treat endometriosis-related infertility is by excision of the endometriotic lesions, which heightens the risk of further adhesion formation in the future. Laparoscopic surgery can improve pregnancy outcomes in some cases, but there is no predictive marker to determine, with certainty, a successful pregnancy. In a retrospective study[43], 194 women with endometriosis underwent laparoscopic surgery, and pregnancy rate was determined after 36 months. The cumulative pregnancy rate 36-months post-surgery was 46.6% (Stage I, 53.6%; stage II,

36.0%; stage III, 51.7%, and stage IV, 41.7%), showing no difference between severity of disease.[43] Another clinical study of 729 women with endometriosis showed the cumulative probability of pregnancy at 3 years from surgery in 537 infertile women was 47% (51% at stage I, 45% at stage II, 46% at stage III and 44% at stage IV).[44]

The ideal treatment should be able to cure the disease rather than suppress development, the side effects should be acceptable, it should be able to treat both pain and enhance fertility, and be affordable and safe for long term use.[36] While many advances in medical therapy for endometriosis have been made over the years, identification of novel drug targets, finding markers for diagnosis, prognosis and response to treatment would be invaluable to the development of future therapies.

1.3 Challenges with current diagnoses and classification systems

Presently there is no single effective diagnostic test for endometriosis. Sadly, women with endometriosis wait an average of 6-12 years before a definitive diagnosis is achieved.[3] This delay is in part due to the reluctance of clinicians to resort to laparoscopic surgery prior to medical management of symptoms[1,10,45]. Although the introduction of laparoscopy has improved the rate of diagnosis, provides better visualization of the peritoneal cavity, and currently holds the title of "gold standard", there still exists limitations with its use as a diagnostic tool.[46] The variability in clinical presentation, the high frequency of asymptomatic women, and the poor correlation between clinical symptoms and stage of disease, make it difficult to diagnose based solely on presenting symptoms.[47,48]. Furthermore, laparoscopic surgery is not inexpensive, and is associated

with risks involving the use of general anaesthesia and potential of adhesion formation post-surgery.[46,49] The use of a serum biomarker for the diagnosis of disease is appealing due in part to the reduced risk of complications that are associated with laparoscopy.

Another issue with laparoscopy is the diagnosis is widely subjective and depends on the surgeon's opinion. At laparoscopy, endometriosis is most commonly classified according to the revised American Society for Reproductive Medicine (rASRM) classification system[50]. This system categorizes disease in four stages depending on location of the lesion and extent of its growth: stage I (minimal), stage II (mild), stage III (moderate), and stage IV (severe). Although it is widely used, it has faced much criticism due to its inability to correlate symptoms with stage of disease. Furthermore, it fails to associate laparoscopic staging, severity of disease, and response to treatment[51,52]. Therefore, this classification system has lacked value in clinical practice.[53,54]

Newer classifications systems such as the Enzian system and the Endometriosis Fertility Index (EFI) have been proposed, but are not as widely used as the rASRM classification system[51].

1.4 Clinical Markers for the Diagnosis of Endometriosis

Endometriosis is a multifaceted disease in which numerous disease pathways are known to be dysregulated.[55] Identification of key signalling molecules in these pathways provide insight for potential therapeutic intervention and diagnosis. The regulation of numerous proteins from different disease pathways have been shown to be dysregulated in women with endometriosis compared to control populations[56–60] (***Fig. 2***). However, the

literature has largely yielded equivocal results[25,61]. Studying pathways involved in apoptosis, immune surveillance, cell adhesion, proteolysis, and others is crucial in understanding disease pathogenesis. It is also important to consider at what phase of disease these pathways are dysregulated. For example, there is an initiation phase when the lesion implants into an ectopic site, followed by a hormone-dependent proliferative phase in which the lesion begins to grow and establish a blood supply. To make matters more complex, these conditions may differ depending on the lesion type and anatomical location of the lesion. The literature has attempted to identify certain markers that may be dysregulated in the above processes.

A robust literature has developed describing potential markers of endometriosis used either alone or in panels for the diagnosis of endometriosis (**Table II**). Ideally, a biomarker for endometriosis should inform physicians about lesion type, prognosis and response to treatment.

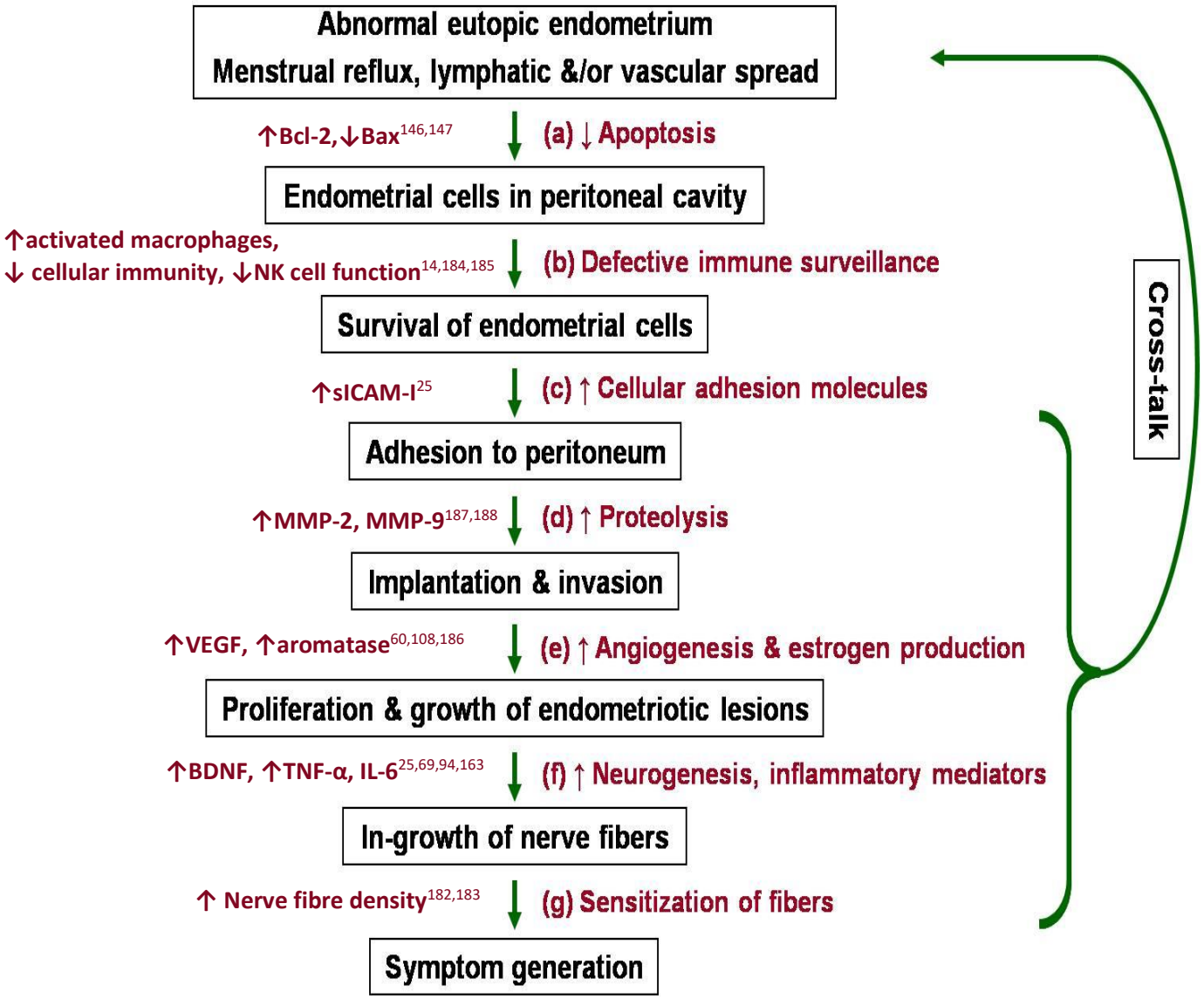


Figure 2. Pathways dysregulated in endometriosis. Figured adapted from Hey-Cunningham et al, 2013[55]. *Bcl-2= B-cell lymphoma 2; Bax= Bcl-2-associated X protein; NK cell= natural killer cell; sICAM-I= soluble intracellular adhesion molecule-I; MMP=matrix metalloproteinases; VEGF= vascular endothelial growth factor; BDNF= brain-derived neurotrophic factor; TNF-α= tumour necrosis factor α; IL-6= interleukin-6.*

Table II. Previously studied circulating biomarkers for endometriosis.

Category	Candidates	Challenges
Glycoproteins[25,61,62]	CA-125	- Elevated in other gynecology malignancies like ovarian cancer; - Low SN: unable to rule in disease at early stages
	CA 19-9	- Similar SP and lower SN than CA-125
	Follistatin	- Superior SN and SP to CA-125, but not reproducible
	Glycodelin A	- Elevated only in serum of women with ovarian endometriosis
Angiogenesis[25,61]	VEGF	- Multiple studies reported no changes in serum VEGF
	Angiogenin	- Elevated in serum of cases only during follicular phase
	HGF	- Levels correlated with disease stage (elevated in late stages), not reproducible
Inflammation[25,61]	IL-6	- Results are conflicting due to methodological differences and lack of studies on early stage disease
	IL-8	- Differences in study approach have rendered conflicting results
	TNFα	- Elevated serum levels in endometriosis, not always reproducible
Cell adhesion and invasion[25,61]	sICAM-I	- Rises in early stages, decreases in later stages of disease
	MMP-2	- Elevated in endometriosis; correlated with later stage disease
Proteomics[25,61]	BDNF	- Elevated in earlier stage disease, decreases with hormonal treatment and removal of lesions
	Protein peaks	- Different studies identify different peaks; cost and time of the technology is a hindrance
Metabolomics[61]	Multiple metabolites	- Groups of metabolites dysregulated together; more research needed to determine their usefulness as diagnostic markers

SN= sensitivity; SP= specificity; CA-125, CA 19-9= cancer antigen; VEGF= vascular endothelial growth factor; HGF= hepatocyte growth factor; IL-6, IL-8= interleukin; sICAM-I=soluble intercellular adhesion molecule 1; MMP-2= matrix metalloproteinase 2; BDNF= brain-derived neurotrophic factor.

1.4.1 Glycoproteins

As previously mentioned, laparoscopic surgery remains the current gold standard for diagnosis, with a sensitivity of 94% and a specificity of 79%[63]. Current alternatives to laparoscopy have been the use of glycoproteins such as cancer antigen 125 (CA-125), which has received the most attention over the years, despite its lack of utility.[61,64–66] Although many studies have demonstrated its correlation to disease severity, it is also an elevated tumour marker in ovarian cancer and thus lacks adequate sensitivity and specificity to serve as a useful clinical marker of endometriosis.[67,68] CA-125 can be detected in disease at later stages (stage III-IV), but not early stage disease (stage I-II), limiting its use to detect disease severity, but not disease itself.[69] Other glycoproteins such as follistatin, an inhibitor of activin, and glycodelin A, a promotor of neovascularization and cell proliferation, lack reproducibility.[25,56,70]

1.4.2 Inflammatory markers

Inflammatory and immunological markers have also demonstrated an association with endometriosis, and have been considered as potential candidates for biomarkers of disease. Interleukin -1 (IL-1), tumour necrosis factor-α (TNF- α) and interferon-γ (IFN-γ), and Regulated on Activation, Normal T Expressed and Secreted (RANTES), among others, have shown potential, but are not considered an optimal choice due to the inability of the marker to distinguish between endometriosis and other pelvic pathologies.[59,67,71] No consensus has been reached on whether cytokines are suitable as a diagnostic marker for endometriosis.[61]

1.4.3 Angiogenic markers

Markers involved in angiogenesis have also been a popular area of research due to their importance in lesion development and sustenance.[72] Vascular endothelial growth factor (VEGF), hepatocyte growth factors (HGF), and platelet-derived growth factor, among others, have all been explored, but have failed to demonstrate consistent results in the literature.[59,61,73,74] In two studies, serum VEGF levels were reduced after surgical excision of the endometriotic lesion, compared to pre-surgery serum levels.[57,75] Contrary to these results, medical treatment of endometriosis resulted in an increased plasma VEGF measurement in endometriosis patients.[35] To date, no angiogenic markers have shown enough promise to pursue as a biomarker for endometriosis.[61]

Markers of oxidative stress, cell adhesion and invasion, hormones, and many others have been investigated, but the issue of reproducibility arises once again.[61] Although some groups have showed promise by using a panel of biomarkers[76,77] to use as a diagnostic marker, none have reached acceptable levels of sensitivity and specificity, but may still serve as novel drug targets[64,76]. More recently, researchers have adapted new techniques such as proteomics, metabolomics and genomics to attempt to identify any changes between healthy women and those with endometriosis. These study designs allow for a broader approach to the underlying mechanisms of disease.

1.5 Contemporary searches for novel markers

The search for novel markers for the diagnosis of endometriosis has become a high priority research activity[78,79] with activity in several areas including phenomics, genomics, proteomics, and metabolomics showing promise.

1.5.1 Phenomics

The study of phenomics is associated with the physical and biochemical changes that occur in response to environmental influences. Given that endometriosis is likely influenced by environmental effects on gene expression and function, the study of phenomics has become important in identifying differences in women with disease. In a review study[80], the association between body mass index (BMI) and endometriosis in adults (11 studies), and in early life (5 studies) was studied. While a modest inverse correlation was found in the adult population, a stronger inverse correlation was determined between endometriosis in the early life BMI, even after confounding factors were taken into consideration. This study also noted that women with a phenotype of freckles, benign skin growths, and a higher sensitivity to sun exposure was more frequent in women with endometriosis[80]. Another study confirmed that BMI and endometriosis were inversely correlated, for women with ovarian endometriomas and/or deep-infiltrating lesions[81]. More specifically, lower BMIs were more likely to be associated with deep-infiltrating endometriosis[81]. No correlation between peritoneal lesions and BMI was identified.

1.5.2 Genomics

The use of genomics techniques, namely next-generation sequencing (NGS) has become a very powerful tool. Next generation sequencing allows millions of DNA strands to be sequenced simultaneously. NGS has been used in the study of endometriosis to identify changes in the expression of noncoding RNA[82,83].

Epigenetics, the study of modifications to gene expression without altering genetic code, is a branch of genomics that can provide information on environmental effects on disease. Epigenetic regulation includes DNA methylation and histone modifications; these changes can distinguish between different states of disease.[84] Using a genome-wide methylation array on the eutopic endometrium of 7 cases and 6 controls, one group identified 59 hypermethylated genes and 61 hypomethylated genes in women with endometriosis compared to controls.[85] Genes that were not previously associated with endometriosis were differentially methylated suggesting that they play a role in the abnormal regulation of endometrial cell proliferation in endometriosis.[85] RNA sequencing, small nucleotide polymorphism (SNP) arrays and miRNA microarrays, all allow for the detection of sequences or polymorphisms that may be differentially expressed in women with endometriosis compared to a control population.[86–91] Using microarrays with subsequent RT-qPCR validation has been widely used in the search for novel dysregulated miRNA in endometriosis.

1.5.3 Proteomics

Proteomics is an extensive study of proteins including their expression, localization, functions, post-translational modifications and interactions.[92] Proteomic techniques have allowed researchers to simultaneously study hundreds of proteins, especially in the pursuit of a disease biomarker. Proteomics analyses can and have been performed on tissue samples, plasma, serum, peritoneal fluid, urine and menstrual blood.[92]

Protein microarrays have been used in the eutopic endometrium of women with endometriosis and controls.[93] In this study protein microarray identified neurotrophins nerve growth factor (NGF), neurotrophin-4/5 (NT-4/5) and brain-derived neurotrophic factor (BDNF) to be elevated in women with endometriosis. NT-4/5 and BDNF were subsequently validated with ELISA and western blot analysis.

Further analysis with plasma BDNF levels have shown BDNF to be elevated in women with minimal-mild endometriosis prior to surgery; following excision of the lesions, BDNF levels were indistinguishable from asymptomatic controls.[94] Plasma BDNF concentrations were also significantly elevated in stage I-II compared to symptomatic controls and stage III-IV individuals, while NT-4/5 and NGF levels showed no change.[69] Women on hormonal therapy for endometriosis exhibited plasma BDNF levels similar to controls.[69] Finally, in a study from earlier this year, concentrations of pre-operative plasma BDNF were higher in women with ovarian endometrioma compared to other lesion types and controls and was correlated with pain.[95] Taken together, these data highlight the importance of BDNF in the pathology of endometriosis.

Many groups have attempted to elucidate specific peptide and protein patterns by using mass spectrometry. In 2012, one group was able to predict minimal to mild stage endometriosis from a pattern of five peptide and protein peaks discovered by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis on 254 plasma samples (165 cases, 89 controls).[96] Minimal to mild endometriosis could be predicted with 75% sensitivity and 86% specificity, while using another five protein peaks could help predict moderate to severe disease with 98% sensitivity and 81% specificity.[96] The peak with the highest intensity was identified as fibrinogen β-chain peptide.[96] Another group, using the same technique on serum samples, was able to reach 87% sensitivity and 97% specificity for their diagnostic algorithm based on two protein peaks.[97] Interestingly, significant alterations of one of the peaks was observed one month after surgery, suggesting it may have responded to decreased lesion activity.[97] These protein peaks were not identified as any specific protein, and was therefore not pursued further.

1.5.4 Metabolomics

Metabolomics studies try to identify the chemical "fingerprint" resulting from multiple cellular processes. Using proton nuclear magnetic resonance spectroscopy, one study measured the metabolite profiles in the serum of 135 women (75 cases, 60 controls).[98] Women with endometriosis displayed significantly higher reactive oxygen species, lipid peroxidation, and advanced oxidation protein products, as well as significantly lower total antioxidant capacity, superoxide dismutase, catalase, and

glutathione.[98] Another study, using the same approach, identified increased valine, fucose, choline-containing metabolites, lysine/arginine, and lipoproteins, and decreased creatinine levels in the plasma of women with endometriosis (n=50) compared to controls (n=23).[99] Whether these metabolic profiles can be used for diagnostic purposes remains to be determined.

While the studies of phenomics, proteomics, and metabolomics have brought forth new technologies that have since proven to be important in the search for novel markers whose expression is dysregulated in women with endometriosis vs controls, the study of microRNAs remains the most interesting, and promising to pursue further.

1.6 MicroRNAs

Recently, microRNAs (miRNAs) have become a topic of interest in endometriosis research. These short, non-coding RNA strands play a role in the posttranscriptional regulation of genes by either repressing translation or initiating degradation of the mRNA transcript.[88] The regulation of miRNAs is important to maintain proper cellular function and differentiation.[100] Abnormal miRNA expression has already been attributed to benign gynecological conditions, gynecological malignancies and fertility disorders in women.[101] They are stable in biological fluids, as they are resistant to RNase degradation, and are highly expressed and conserved among species.[7] Compared to the amount of mRNA and protein in the cell, the amount of miRNA is limited.[102] However, miRNAs have the ability to regulate hundreds of mRNAs and therefore have considerable effects on gene expression networks.[102] Several studies have therefore focused on using miRNAs as a

potential diagnostic tool for endometriosis. Analyses of serum[88,103–105], plasma[90,106,107], endometrial tissue[87,89,91], and endometriotic implants[60,87,108] have all been performed in women with and without endometriosis.

1.6.1 Biogenesis of miRNAs

All small eukaryotic RNAs are classified not only by their size, but also by their association with the Argonaute (AGO) protein family.[109] These proteins are necessary components of the RNA-induced silencing complex (RISC) which is involved in the gene silencing process known as RNA interference (RNAi). In animals, small RNAs are classified into three groups: miRNAs, small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs).[109] In most cases, microRNAs are transcribed by RNA polymerase II (Pol II), otherwise they can be transcribed by Pol III.[110] Transcription of the miRNA transcript results in a several kilobases (kb) long transcript with stem-loop structure and hairpins called primary transcript (pri-miRNA).[110] The hairpin structures are then cleaved at the stem to release what is now the short "pre-miRNA" structure.[110] This cleavage is performed in the nucleus by the nuclear RNase-III type protein Drosha, which is around 160kDa.[110,111] The rest of the transcript is thought to be degraded in the nucleus.[111]

Further processing occurs in the cytoplasm, therefore the pre-miRNA needs to be exported from the nucleus for this to begin. Exportin-5, a nuclear transport receptor, is responsible for the translocation of the pre-miRNA transcript.[111] In the cytoplasm, the pre-miRNAs are processed into 22 nucleotide duplexes by the RNase III protein Dicer,

which is also known to play a role in processing siRNAs.[110,111] Once the miRNA duplex is generated, it is loaded onto an AGO protein to generate the RISC, which also contains Dicer and TAR RNA-binding protein (TRBP).[110] In the duplex, one of the strands remains bound to AGO as a guide strand, while the other strand (the passenger strand, often denoted with an *) is degraded by an RNA helicase.[110] The relative thermodynamic stability of the two ends of the RNA duplex determines which strand is the mature strand and which one is the passenger strand.[109–111] The strand with the more unstable 5' end is usually selected as the guide strand, but this is not a strict process.[109] Sometimes, the passenger strand can be selected and can act in silencing gene transcripts, however it is not as potent as its corresponding guide strand.[109] These processes do not go as smoothly as it seems; steps at the transcriptional level, post-transcriptional level and multiple feedback loops in the miRNA network affect the production of miRNAs.[110]

1.6.2 Functions of miRNAs

The function of miRNAs is to post-transcriptionally regulate gene expression by binding to complementary sequences in the coding or 3' UTR of their target messenger RNA (mRNA).[7,101,109] Within the RISC, the miRNA binds to its targets with its seed sequence, the nucleotides in position 2-8 near the 5' end of the transcript.[101] Perfect binding between the seed sequence and the 3' UTR of the mRNA target leads to mRNA degradation, while slightly imperfect binding can lead to translational repression.[7] When miRNAs share the same seed sequence, they are a part of the same miRNA family and they share similar target genes.[112] Although the majority of the literature discusses

miRNAs as inhibitory, there is some evidence of miRNAs enhancing translation through modulations in the AGO proteins in the RISC.[113] miRNA can exert their effects on post-transcriptional gene regulation in three different ways: translational inhibition, translational enhancement, and mRNA degradation.[101]

As well as acting locally, it has been suggested that miRNAs elicit their functions in distal cells/organs through the circulation.[7] Microvesicles, exosomes and complexes with lipoproteins allow miRNA to remain stable in the cell-free form. miRNA can exert a similar biological function to recipient cells once taken in by either endocytosis or by cell surface receptor recognition of the complex to which miRNA is bound.[7] Because miRNAs exist in biological fluid they have become an ideal target as disease-specific biomarkers. The dysregulation of miRNAs is currently being studied in many diseases including cardiovascular [114], ageing-related [115], cancers[116–119], and gynecological diseases[101]. Because miRNAs have hundreds of targets, and each target may be regulated by hundreds of miRNA transcripts, the relationship between miRNA and disease is complex. When studying miRNA expression, the three most commonly used methods are: RT-qPCR, microarray hybridization, and next-generation sequencing (NGS); all of which have their advantages and disadvantages.

1.6.3 Circulating miRNAs

Several studies have investigated the expression of circulating miRNAs in women with endometriosis, but have so far yielded inconsistent results (**Table III**). In some of these studies, differential expression of miRNAs were first determined by miRNA

profiling analysis and were further validated by quantitative real-time polymerase chain reaction (qPCR).[88,91,106] Jia et al identified 27 miRNAs in their array (>2-fold difference), with only a fraction of those validated by qPCR in the plasma of their study subjects.[106] Significant downregulation was observed in only three of these miRNAs (miR-17-5p, miR-20a, and miR-22) in women with endometriosis by 5.4-, 4.5-, and 2.6-fold, respectively.[106] The abnormal expression of these miRNA's ultimately affects the post-transcriptional regulation of their targets including anti-apoptotic protein B-cell

Table III. Studies measuring changes in circulating miRNAs.

Study	miRNA	Sample size	Sample type	Screening method	Reference gene	Disease stage
Wang et al, 2012[88]	↑: miR-199a, miR-122 ↓: miR-145*, miR-141*, miR-9*	60 cases, 25 controls (symptomatic)	serum	Microarray, RT-qPCR validation	RNU6	Stage I +II: 22; Stage III+IV: 38
Jia et al, 2013[106]	↓: miR-17-5p, miR-20a, miR-22	23 cases, 23 controls (symptomatic	plasma	Microarray, RT-qPCR validation	miR-16	Stage I+II: 0 Stage III+IV: 23
Rekker et al, 2015[107]	↓: miR-200a, miR-141	61 cases, 30 controls (healthy)	serum	RT-qPCR	miR-30e-5p, miR-99a-5p	Stage I+II: 33 Stage III+IV: 28
Cosar et al, 2016[120]	↑: miR-125b, miR-451a ↓: miR-3613	24 cases, 24 controls (symptomatic)	serum	Microarray, RT-qPCR validation	RNU6	Stage I+II: 0 Stage III+IV: 24
Hsu et al, 2014[103]	↓: miR-199a	40 cases, 25 controls (symptomatic	serum	Microarray, RT-qPCR validation	N/A	N/A
Cho et al, 2015[104]	↓ miR-let-7b, miR-135a	24 cases, 24 controls (symptomatic)	serum	RT-qPCR	RNU6	Stage I+II: 0 Stage III+IV: 24
Suryawanshi et al, 2013[90]	↑: miR-16, miR-191, miR-195	33 cases, 20 controls (healthy)	plasma	Global profiling, and RT-qPCR	miR-132	N/A

RT-qPCR= Real-time quantitative polymerase chain reaction. miRs in red have been dysregulated in more than one study.

CLL/lymphoma 2 (Bcl-2), interleukin-8 (IL-8), hypoxia-inducible factor -1α (HIF-1α) and VEGF-A among others.[106]; all of which have been shown to be dysregulated in women with endometriosis compared to disease free controls. In another study on plasma samples, Rekker and colleagues noted significant changes in miRNA expression (specifically miR-141, miR-200a, and miR-200b) depending on the time of day the sample was retrieved.[107] The miRNA levels were lower in the morning than in the evening for all samples (subjects with endometriosis, laparoscopically confirmed endometriosis-free women, and self-reported healthy women).[107] All three miRNAs were found to be as significantly different in morning samples of endometriosis free and healthy women, and miR-200a levels were 1.7x lower in women with endometriosis compared to endometriosis-free controls.[107] This study identifies another major roadblock in endometriosis research with respect to miRNA expression; if miRNA expression is regulated by the circadian clock, what portion of change is attributed to endometriosis?

Changes in miRNA expression have been identified in serum of women with endometriosis. Wang et al determined that the relative expression of miR-199a (1.0×10^5-fold) and miR-122 (4.2×10^5-fold) were significantly up-regulated in endometriosis samples compared to controls, whereas miR-145*, miR-141*, miR-542-3p, and miR-9* were significantly down-regulated by 3.5×10^3-, 2.8×10^4-, 10.4-, and 3.9×10^3-fold, respectively.[88] Furthermore, miR-199a and miR-122 expression discriminated between severity of endometriosis, and aberrant miR-199a expression was found to be associated with the chronic pelvic pain of endometriosis.[88] Contrary to this study, another group found miR-199a to be significantly down-regulated by 1.5x in the serum of women with

endometriosis compared to controls[103]. Significant down-regulation of circulating let-7b and miR-135a was found in the serum of women with endometriosis compared with controls (3.2- and 2.0-fold, respectively), while let-7d and 7f exhibited a non-significant trend towards a decrease.[104] Interestingly, let-7b expression correlated strongly with serum CA-125 levels, a protein formerly considered a potential biomarker for endometriosis.[104] Further analysis based on menstrual cycle phase revealed significantly lower expression of let-7b, -7c, -7d, and -7e in women with endometriosis during the proliferative phase.[104] In 2016, Cosar and colleagues provided the most promising results thus far.[105] This group identified three miRNAs (miR-125b-5p, miR-451a, and miR-3613-5p), in serum samples via microarray profiling, that when used together could provide both a sensitivity and specificity of 100% in diagnosing endometriosis.[105] This study however, along with many other studies in endometriosis research, did not include women with earlier stages of disease (stage I-II). The diagnostic markers proposed in this study may therefore not be applicable to all women with endometriosis, but limited to only those with moderate to severe disease. Obtaining participants with minimal or mild disease is a challenge researchers undoubtedly face, as many of these women are unlikely to go through laparoscopic surgery for their symptoms. Introducing disease stage and menstrual cycle phase into consideration when comparing the abnormal expression of these miRNAs is highly desirable, but not always feasible since it results in a lower sample size.

1.6.4 MicroRNAs in tissues

Studies using endometrial tissue samples present more of a challenge due to the lack of a consistent study design (**Table IV**). There is the possibility of comparing tissues between ectopic lesions and eutopic endometrium in the same subject, or comparing them with a symptomatic control, or both. Lesion type also changes the way data is interpreted because different lesion types may not be able to express miRNA, or contain cells that are not viable (i.e. white or yellow-brown lesions, indicating latent disease).[12]

Eutopic endometrium of cases vs. controls

Some studies choose to compare the eutopic endometrium of cases to those of controls, instead of studying the miRNA profile of the ectopic lesions. These studies aim to identify differences in the endometrium of health and diseased women that may result in pathology. miR-483, and miR-629* were shown to be significantly downregulated by approximately 2-fold using microarray and further RT-qPCR confirmation in eutopic proliferative endometrium of women with OMA compared to symptomatic controls[121]. In another study, early secretory endometrium of women with different lesion types were compared to the eutopic endometrium of symptomatic women[91]. Using microarray and further RT-qPCR confirmation, they found miR-9, miR-34b, and -34c were significantly downregulated in cases, by 26-, 7- , and 4-fold respectively[91]. Although the study designs were similar, differences in menstrual stage, microarray platforms, normalization methods, sample size, and study population created vastly different results.

Table IV. Summary table of studies measuring miRNA in tissue.

Study	miRNA†	Sample Size	Case tissue	Control tissue	Reference genes	Stage	Screening method
Eutopic case vs Eutopic control							
Laudanski et al, 2015[121]	↓: miR-483-5p, miR-629*	21 cases, 25 controls	Eutopic proliferative endometrium (of women with OMA)	Symptomatic controls	RNU6	Stage I+II: 0 Stage II+IV: 21	Microarray, RT-qPCR validation
Burney et al, 2009[91]	↓: miR-9, miR-34b, miR-34c	4 cases, 3 controls	Eutopic secretory endometrium (of women with OMA, PL and DIE)	Symptomatic controls (leiomyoma)	miR-5S	Stage I+II: 0 Stage III+IV: 4	Microarray, RT-qPCR validation
Aghajanova and Giudice, 2011[122]	↑: miR-21	63 cases, 12 controls	Eutopic endometrium	Symptomatic controls	N/A	Stage I+II: 19 Stage III+IV: 44	microarray
Ectopic tissue vs Eutopic endometrium (paired and/or control)							
Zheng et al, 2014[123]	↑: miR-143, miR-145	11 cases, 22 controls	OMA	Paired eutopic endometrium, symptomatic controls (leiomyoma)	RNU6	N/A	RT-qPCR
Shen et al, 2013[124]	↓: miR-23a, miR-23b	23 cases (paired ectopic and eutopic), 15 controls	OMA	Paired proliferative eutopic endometrium, healthy controls	RNU6	Stage I+II; 0 Stage III+IV: 23	RT-qPCR
Liu et al, 2012[125]	↓: miR-126	31 cases (31 eutopic, 16 ectopic), 27 controls	OMA	Paired eutopic endometrium, symptomatic controls	RNU6	Stage I+II: 6 Stage III+IV: 25	RT-qPCR
Long et al, 2015[126]	↓: miR-29c	20 paired ectopic/eutopic, 10 controls	Not specified	Paired eutopic endometrium, symptomatic controls	RNU6	N/A	RT-qPCR

Ohlsson-Teague et al, 2009[87]	↑: miR-145 ↓: miR-141	8 (ectopic and eutopic sample from each)	PE	Paired eutopic endometrium	miR-let-7a, miR-let-7d	Stage II-IV (no specifics)	Microarray, RT-qPCR validation
Filigheddu et al, 2009[127]	↑: miR-202-5p ↓: miR-200a, miR-200b, miR-200c	16 (ectopic and eutopic sample from each)	OMA	Paired eutopic endometrium	RNU18	Stage I+II: 0 Stage III+IV: 16	Microarray, RT-qPCR validation
Hawkins et al, 2011[128]	↑: miR-29c ↓: miR-200b	18 cases, 20 controls	OMA	Symptomatic controls	Human ribosomal gene L19	N/A	NGS, RT-qPCR validation
Comparisons between endometriotic lesions							
Braza-Boïls et al, 2009[60]	↑: miR-411, miR-29c-3p, miR-138, miR-424 ↓: miR-202-3p, miR-449b-3p, miR-556-3p	51 cases, 32 controls	51 OMAs with 18 PE or 20 DIE	Paired eutopic, Asymptomatic controls	RNU6	N/A	Microarray, RT-qPCR validation
Ramón et al, 2011 [108]	↑:miR-125a, miR-222 (OMA) ↓: miR-17-5p	41 cases, 38 controls	41 OMAs with 24 PE, and 13 DIE	Paired eutopic, Histologically-confirmed controls	RNU6	N/A	RT-qPCR
Graham et al, 2015[129]	↑: miR-451	43 cases, 30 controls	PE, OMA, and DIE	Paired eutopic endometrium	RNU6	Stage I+II: 8 Stage III+IV: 22	RT-qPCR
Ectopic tissue vs. Adjacent peritoneum							
Saare et al, 2014[130]	↑: miR-449a, miR-34c, miR-200a, miR-200b, miR-141	22 cases, 24 controls	PE	Adjacent peritoneum (of cases)	RNU44, RNU48	Stage I+II: 0 Stage III+IV: 22	RNAseq, RT-qPCR validation

†: up-or down-regulated relative to control groups; PE= peritoneal lesion, OMA=ovarian endometrioma, DIE=deep-infiltrating endometriosis, RT-qPCR= Real-time quantitative polymerase chain reaction. miRNAs highlighted in red have been dysregulated in more than one study.

Ectopic vs. paired eutopic endometrium

Another common method of measuring miRNA differences is by comparing normal endometrium and endometriotic tissue within the same woman. Ohlsson-Teague et al assessed miRNA expression in peritoneal lesions (PE) and paired eutopic endometrial tissue.[87] They identified 14 up-regulated (including miR-20a, miR-99a, miR-126, and miR-145), and 8 down-regulated miRNAs (including miR-141, miR-200b, and miR-424) with a fold-change of at least ±1.5 times.[87] In this study, miR-let-7a and -7d were used as control miRNAs as opposed to potential biomarkers.[87]

Another study, compared ectopic OMA tissue to paired eutopic endometrium[127]. Using microarray and further RT-qPCR confirmation, this group found a significant increase in miR-202 expression (9-fold) and downregulation of miR-200a, -200b, and -200c, in ectopic tissue (18-, 26-, and 18-fold, respectively)[127]. Using only RT-qPCR, one group measured miR-143, and miR-145 to be upregulated in OMA compared to paired endometrium by approximately 2.8-fold[123]. These three studies had different proportions of women in each stage of disease, if noted at all[123]. Many other studies have been conducted in a similar manner, more often than not producing different results (**Table IV**).

miRNA expression in endometriotic lesions

Of the three most common comparisons when studying miRNA expression in endometriosis, comparing expression between lesion types is the least performed. Perhaps this is due to the difficulty in obtaining samples, or uncertainty that the samples contain

endometriotic tissue. One group from Spain performed two separate studies on differential miR expression in endometriotic lesions, with a larger focus on miR expression in OMAs[60,108]. Ovarian endometriomas were likely favoured because all the cases in the study had OMAs, and the PE and DIE groups were subsets from the OMA group. miR-16 was significantly upregulated in DIE (6.2-fold) and PE (3.6-fold) compared to OMA[60]. Furthermore, miR-29c was significantly higher in DIE compared to OMAs (2.2-fold), and significant downregulation of miR-202 was observed in DIE compared to OMAs (7.1-fold). These miRNAs, along with many others were differentially regulated in these studies[60,108], confirming that these lesions are biochemically distinct, even if from the same woman.

1.6.5 Relationship with biological function relevant to endometriosis pathology

An association has been made between angiogenesis and the following miRNAs: miR-222, miR17-5p, miR-424, miR-449b-3p, and miR-126.[60,108,131,132] Further studies have identified a link between angiogenesis and endometriosis.[72,133,134] Ramón et al used paired eutopic and ectopic endometrial samples to compare expression of specific miRNAs and proteins associated with angiogenesis.[108] In ovarian endometrioma, significant upregulation of antiangiogenic miRs, miR-21 (1.7x) , miR-125a (2.1x) and miR-222 (2.5x) compared to eutopic endometrium was observed, as well as significantly downregulated expression of proangiogenic miRs, miR-20a (1.5x) and miR-17-5p (1.8x).[108] Furthermore, ovarian endometrioma had significantly higher expression of thrombospondin-1 (TSP-1), an angiogenic inhibitor, compared to eutopic endometrium,

and no change in vascular endothelial growth factor (VEGF) in these two groups.[108] Further examination between lesion type (ovarian endometrioma, peritoneal lesion, and rectovaginal lesion) were assessed additional differences in expression were observed whereas menstrual cycle phase (secretory and proliferative) did not significantly affect expression.[108]

1.6.6 Challenges in miRNA research

Using miRNAs to understand endometriosis continues to face numerous hurdles. Inconsistent data are often acknowledged, but are not always addressed. Study participant characteristics are different between studies. A consistent control group has yet to be agreed upon between researchers, with some using paired eutopic endometrium[60,87,123,127], control endometrium from symptomatic[91,122,125,128] or asymptomatic[60,124] women, and even adjacent peritoneum[130]. Many studies also lack a sufficient population of women with minimal to mild stage of disease, mainly because these women often go undiagnosed, making it more difficult to study differences between disease severity. These issues, along with the reality that most studies lack a diverse study population, make replication of study results a challenge.

Methodological differences are also a major contributor to varied results. The majority of studies identifying changes in miRNA expression use microarray technology to do so. Therefore many of the microarray results are not validated, and microarray findings between studies are vastly different. Differences in protocols, amount of miRNA used, primers, and cycling conditions can vary widely between studies. Ultimately, we are

left with the discovery of many miRNAs that are not able to detect endometriosis in a wider study population. Furthermore, the use of a universal reference gene to normalize has also posed a problem, as the most commonly used reference gene, RNU6[60,89,123,124,126,129,135], is considered unstable[136]

The majority of studies fail to recognize endometriosis as a heterogeneous disease, which poses another challenge. For example, biochemical characteristics may be affected by factors such as lesion type (clear, red, or blue-black), location (endometrioma, peritoneal, or deeply-infiltrating), duration of disease, history of surgery, BMI, and comorbidities. While looking for an individual biomarker has been a struggle, the search for panels of biomarkers has begun. While microRNAs are a promising areas of investigation[105], there are some limitations. Currently there are no associations between patient characteristics, severity of symptoms, lesion type, or stage of disease[52]. Therefore, evaluation of miRNA expression by anatomical location may provide further insight into potential drug targets, as well as providing surgeons with information about the manifestation of disease prior to surgery. Seeing as endometriosis presents itself so differently between individuals, I suggest that this information can potentially help physicians achieve individualized patient care.

1.7 Hypotheses

Current therapeutic options remain suboptimal and the difficulty in diagnosing endometriosis has significantly delayed the time taken to treat women with disease, negatively impacting their quality of life. The study of disease pathways for identification

of novel therapeutic targets and diagnostic markers has emerged as a high priority research topic. Current literature on miRNAs shows promise, but inconsistencies in the literature due in part to the lack of standardized methodologies remain.

Consequently, I hypothesize the following:

1. miRNA expression will differ in cases (women with surgically confirmed endometriosis) versus controls (surgically confirmed not to have endometriosis)
2. miRNA expression will differ depending on lesion type—peritoneal lesions, ovarian endometriomas, and deeply-infiltrating endometriosis.
3. miRNA associated with BDNF expression will be lower in cases vs controls.

1.8 Main Objectives

Current literature on the diagnostic potential of miRNAs in women with endometriosis is inconclusive. The majority of the results generated from these studies have not been reproducible, and no two studies have been able to identify and confirm the differential expression of the same group of miRNAs. Potential reasons include differences in study design, study populations, and analytical methods used, along with other considerations such comorbidities and burden of illness, which differs for each woman. I postulate that endometriosis is a heterogeneous disease and caution must be taken when grouping together all women with endometriosis compared to women without endometriosis, since women with differing manifestations of disease may express individual miRNA differently. Similarly, I postulate that different lesion types will demonstrate differential expression of miRNA.

Therefore, the main objectives of the study are to:

I. Identify differential expression of miRNAs in eutopic endometrium of cases compared to symptomatic controls, as well as changes in miRNA expression between endometriotic implants.

Specific Aims

1. Histological confirmation of disease status;
2. Identify miRNAs that are prevalent in the endometriosis literature;
3. Identify a series of reference genes that are stably expression between tissue types, in order to properly normalize miRNA expression;
4. Measure miRNA expression with real-time polymerase chain reaction (RT-qPCR); and
5. Compare miRNA expression in eutopic endometrium from women with endometriosis vs. symptomatic controls using the $\Delta\Delta C_t$ analysis method[137,138]

II. To quantify differences in miRNA expression and determine gene and protein expression in endometriotic lesions from different lesion types.

Specific Aims

1. Select target genes that are regulated by the studied miRNA and that function in processes that promote lesion development (cell proliferation, cell differentiation, angiogenesis, anti-apoptosis, etc.);
2. Measure gene expression of said targets with RT- qPCR; and

3. Normalize to a reference gene that is stably expressed throughout tissue type.

III. Measure miRNA associated with BDNF expression.

Specific Aims

1. Measure protein expression in lesion types using western blot analysis; and
2. Measure target concentrations in the plasma using an enzyme-linked immunosorbent assay (ELISA).

CHAPTER 2: MATERIALS AND METHODS

2.1 *Quantify and compare the relative expression of six microRNAs in eutopic and ectopic tissues of women with and without endometriosis*

2.1.1 Study Participant Recruitment

Forty-seven women were recruited and screened for inclusion in this study. All forty-seven women underwent gynecological laparoscopic surgery between April 2011 and March 2017 for pelvic pain thought to be due to endometriosis. Thirty-eight of these women were found to have pathology-confirmed endometriosis, and the remaining nine women were diagnosed with other gynecological conditions (symptomatic controls). The exclusion criteria for this study were individuals unable to provide consent, under age 18, diagnosed with adenomyosis, heavy smokers, and use of gonadotropin-releasing hormone agonist (GnRH-a) three months prior to surgery.

All participants completed questionnaires on demographics and gynecologic variables. Questions included average length of cycle, age of first menstruation, and medical histories. The stage of endometriosis was determined by the surgeon during surgery according to the revised Classification of the American Society of Reproductive Medicine (rASRM). This study was approved by the Research Ethics Board, McMaster University (Institutional Review Board no. 12-083-T), and all participants provided written informed consent before surgery.

Stage of disease was determined by reading operative reports and using the rASRM guidelines. Pathology reports were obtained to determine if samples were

positive or negative for endometriosis. Other previous data obtained from questionnaires were translated into a database for easier retrieval.

To determine menstrual phase of eutopic endometrium, endometrial samples were fixed by immersion in cold 10% phosphate buffered formalin (pH 7.4). Tissue samples were washed in water, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin. Paraffin sections were cut on a rotating microtome (4-5 μm thick) and stained with hematoxylin and eosin for histological analysis. Histological sections were examined using an Olympus IX81 microscope at 20 and 40x magnification.

2.1.2 Sample Preparation

Endometrium samples were obtained during surgery from study participants (n=38) and control subjects (n=9). The samples were snap frozen in liquid nitrogen for protein analysis, or stabilized with RNA-later (Thermo Scientific, Mississauga, ON) for miRNA analysis.

Thirty milligram sections of each sample were weighed and placed into 700 ul of QIAzol Lysis Reagent before homogenization (Bio-Gen, Oxford, CT). Total RNA and miRNA were isolated from the samples with the miRNeasy mini kit (Qiagen, Toronto, ON), and miRNA concentration and purity was measured with the NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA synthesis was performed on these samples with miScript II RT Kit (Qiagen). Each individual reverse transcriptase reaction consisted of 4ul of 5x miScript HiSpec Buffer, 2ul of 10x miScript Nucleics Mix, 2ul of miScript Reverse Transcriptase Mix, and variable volumes of Template RNA (200 ng) and RNase-

free water, to give a total volume of 20 ul. Reactions were performed in an iCycler Thermocycler (Bio-Rad) for 60 min at 37˚C followed by 5 min at 95˚C to inactivate the enzyme. The samples were later diluted with RNase-free water to a final concentration of 3ng/µl.

2.1.3. Quantification of microRNA expression – Real-time quantitative polymerase chain reaction

The expression levels of Hs_miR-9_1 (cat. no. MS00010752, hereafter referred to as miR-9), Hs_miR-21_2 (MS00009079, miR-21), Hs_miR-141*_1 (MS00008680, miR-141), Hs_miR-200b*_1 (MS00009023, miR-200b), Hs_miR-424_1 (MS00004186, miR-424), Hs_miR-10a_2 (MS00031262, miR-10a), Hs_miR-10b_3 (M00031269, miR-10b), and hsa-miR-204-5p (MIRAP00256, miR-204) were measured using quantitative real-time polymerase chain reaction (PCR) with the use of miScript SYBR Green PCR kit (Qiagen). The expression of these genes were measured against reference genes: Hs_SNORD68_11 (MS00033712, SNORD68), Hs_SNORD95_11 (MS00033726, SNORD95), and Hs_SNORD96A_11 (MS00033733, SNORD96A). The majority of the primers are miScript Primer Assays (Qiagen), with the exception of primers for miR-204, which is a MystiCq® microRNA qPCR assay primer (Sigma, Oakville, ON).

Real-time PCR was performed using the LightCycler480 machine (Roche) in 384-well plates with 10ul in each reaction mixture that included 5ul of the 2x miScript SYBR Green PCR Master-Mix, 1ul of the 10x miScript Universal Primer, 1ul of the (target or

reference) gene, 2ul of RNase-free water, and 1ul of the template cDNA. Reactions were subject to an initial activation step of 95˚C for 15 min, followed by 45 cycles of 94˚C for 15 seconds, 55˚C for 30 seconds and 70˚C for 30 seconds. Each sample was run in triplicate for analysis.

2.2 *Quantification of BDNF in plasma and endometriotic lesions*

2.2.1 BDNF mRNA

Endometrial samples were prepared as previously mentioned for miRNA extraction. RNA was extracted from each sample at the same time miRNA was extracted, using the miRNeasy® Mini Kit (Qiagen, cat. no. 217004), according to the manufacturer's protocol, resulting in 40ul of eluate. Following RNA purification, RNA concentration and purity (260/280) were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription reactions were carried out with iScript™ cDNA Synthesis Kit (Bio-Rad). Briefly, each 20 ul RT reaction was made up of 4ul 5x iScript reaction mix, 1ul iScript reverse transcriptase, and variable amounts of Nuclease-free water and RNA template necessary to achieve 500ng of RNA template. Each reaction tube was subject to 25˚C for 5 minutes, 42˚C for 30 minutes, 85˚C for 5 minutes, followed by an optional 4˚C hold.

The cDNA used for qPCR was diluted 1:100 (1ul of stock cDNA in 99ul RNase-free water). The qPCR was run using LightCycler® 480 SYBR Green I Master (Roche, Mississauga, ON). Each 10ul reaction (per well) was composed of 5ul SYBR Green I

Master, 2x concentrated, 1.5 ul Nuclease-free water, 0.5ul forward primer, 0.5ul reverse primer, and 2.5 ul cDNA template.

The primer sequence for BDNF is as follows: forward: 5'-GAG CTG AGC GTG TGT GAC AG -3', reverse: 5'- CTT ATG AAT CGC CAG CCA AT-3', as previously used for Wessels, et al.[69] The stably expressed glyceraldehyde-3-phosphate (GAPDH; Hs_GAPDH_1_SG QuantiTect Primer Assay) was used to normalize gene expression data in this study (Qiagen, cat. no. QT00079247).

Real-time PCR was performed using the LightCycler480 machine (Roche) in 384-well plates with 10ul in each reaction mixture that included 5ul of the LightCycler®480 SYBR Green I Master (Roche, Mississauga, ON.), 0.5ul both the forward and reverse primers, 1.5 ul of RNase free water and 2.5ul of the template cDNA. Reactions were subject to an initial activation step of 95˚C for 5 min, followed by 50-60 cycles of 94˚C for 10 seconds, 60˚C for 10 seconds and 72˚C for 10 seconds, followed by one cycle for melting curve acquisition. This cycle subjected the reactions to 5 seconds at 95˚C, and 1 minute at 65˚C. Each sample was run in triplicate for analysis

2.2.2 BDNF protein

Endometrial biopsies and ectopic implants were snap frozen in liquid nitrogen and stored at -80˚C for future analysis. Approximately 15 mg of sample was placed into 300ul of RIPA Lysis and Extraction buffer (ThermoFisher Scientific) on ice, with the appropriate amount of 100x Halt™ Protease and Phosphatase Inhibitor Cocktail

(ThermoFisher Scientific). Samples were homogenized (Bio-Gen, Oxford, CT) using two 10 second pulses on ice. Following homogenization, samples were subject to gentle agitation at 4˚C for two hours. Finally, the samples were centrifuged for 20 min at 12,000 rpm at 4˚C (Sorvall™ Legend™ Micro 17R Microcentrifuge, ThermoFisher Scientific). The supernatant was stored for future use at -20˚C. Protein extracts were prepared (diluted by four with double-distilled water) for measurement using the Pierce BCA Protein Assay Kit (Thermo-Fisher), and were measured in a microplate reader (BioTek Instruments, Winooksi, VT). A pooled sample was made with 10ul from each homogenate.

Ten micrograms of protein were loaded onto a 12% or 10% Mini-PROTEAN® TGX Stain-Free™ gel (Bio-Rad) and run at 50V for 5 min, and 200 V for 40 min. Gel activation using the ChemiDoc XRS+ System and Image Lab™ 6.0 software (Bio-Rad). Transfers were done using the Trans-Blot® Turbo™ Transfer System using low-fluorescence polyvinylidene fluoride (LF-PVDF) membranes (Bio-Rad) for 7 minutes at 2.5 A, 25 V.

Following transfer, membranes were blocked with 5% skim milk in TBST (0.01% Tween-20) for one hour at room temperature, on a rocker. After blocking, membranes were directly incubated with BDNF primary antibody (ab108319, Abcam, Toronto, ON) at 1:1000 in 5% milk in TBST at 4˚C overnight. The following day, the membranes were subject to 6 x 5 min washes with TBST prior to 1 hour incubation of secondary goat-anti-rabbit HRP at 1:10000 in 5% milk in TBST at room temperature while rocking. Following incubation of the secondary antibody, the blots were washed for 6 x5 min

washes with TBS-T, prior to imaging. The blots were then imaged on the "Stain-Free Blot" protocol for an optimized time of 17 second to capture the total protein using ImageLab™ 6.0 software (Bio-Rad). This image is used for normalization of the blot. Blots were incubated for five minutes with Clarity Max™ Western ECL Blotting substrate (Bio-Rad) prior to imaging. Optimal imagine time for bands was 90 seconds. Images were captured using the ChemiDoc XRS+ (Bio-Rad).

2.2.3 Plasma BDNF

Plasma samples were obtained prior to surgery in K2 EDTA BD Vacutainer® collection tubes (Fisher Scientific). The samples were left on ice for about 40 minutes prior to centrifugation at 3000 rpm for 20 minutes in an Eppendorf™ 5702 Series Centrifuge (Fisher Scientific). The supernatant was then aliquoted into Sarstedt Cryopure 1.8ml tubes (Fisher Scientific) and stored at -80˚C.

BDNF was measured in duplicate by using the BDNF E_{max}® ImmunoAssay system, and following the manufacturer's protocol (Promega, Madison, WI.) with a few minor changes. Prior to the experiment, the plasma samples were not acid-treated, but they were diluted 1:10 with 1x sample buffer provided by the kit (Promega). During wash steps, a plate washer was not available, so plates were washed gently with Tris-buffered saline + 0.05% tween-20 (TBST) using a multichannel pipette. The TBST was made fresh, and autoclaved the day before using. Absorbance was read at 450nm on a plate reader within 30 minutes of stopping the reactions.

2.2.4 Statistical Analysis

Demographic variables such as age, ethnicity, and menstrual stage were measured as frequency and percentage, with the exception of age, which was calculated as mean (±SD). Differences between cases and controls were measured with Mann-Whitney U test, Fisher's Exact tests, or Chi-Squared analysis. Significance was measured at α=0.05.

Relative quantification was performed with SNORD95, SNORD96A and SNORD68 as references genes in OMA samples (n=15), and PL samples (n=11) and DIE samples (n=13) using the LightCycler480 (Roche) software. This software calculates an efficiency corrected normalized ratio of target gene to the references genes using an algorithm developed by the software ($2^{\Delta Ct}$). The individual ratio for each sample was then divided by the average control sample ratio, which follows the $2^{-\Delta\Delta Ct}$method to detect fold change of expression.[138]

Using GraphPad Prism version 5.01 (La Jolla, CA), the Kolmogorov-Smirnov test for normality was used to determine if the data was normal. A Student's t-test or Mann-Whitney U Test was performed to compare miRNA expression of control endometrium and eutopic endometrium of all cases, control endometrium compared to eutopic endometrium of women with different lesions, and finally of ectopic implants and matched eutopic endometrium. A One-Way ANOVA or Kruskal-Wallis Analysis of Ranks was performed between the ectopic implants. Post-hoc analysis was performed using a Tukey's Test or a Dunn's Test. Linear regression analysis was performed on ectopic implants. Data are presented in scatter-plot graphs depicting medians. Significance was denoted as α=0.05.

To analyze mRNA data on tissues, BDNF expression was first normalized to GAPDH. Following normalization, the data was subject to Mann-Whitney U tests or Kruskal-Wallis analysis on ranks to determine differences in BDNF gene expression, similar to analysis of miRNA.

Protein expression was normalized to total protein in each lane, which was captured using the Chemi-Doc (Bio-Rad). Using the ImageLab™ 6.0 software (Bio-Rad), total volume of the band in each lane was adjusted for with total lane protein and by calculating a gel factor that used a pooled sample that was run on every gel. Total normalized volume of the band of interest was compared between sample groups using student's t-tests and one-way ANOVA analyses.

Plasma BDNF was measured using the ELISA kit as previously mentioned. Data was corrected by running the same sample on every plate and adjusting for a differences in each plate run. Plasma concentrations were obtained by the using the standard curve generated by Gen5 software version 2.03.1 (BioTek Instruments). Concentrations were multiplied by 4 (since they were diluted by 4 prior to the experiment), and were compared between groups using a student's t-test or a one-way ANOVA.

CHAPTER 3: RESULTS

3.1 Characteristics of the Study Population

The mean age of controls and cases was 34.7 ± 8.0 and 33.9 ± 4.3 years, respectively and demographic variables such as ethnicity (p=0.288), age of first menses (p=0.436), and duration of bleeding (p=0.830) did not differ between cases and controls (**Table V**). Most cases (84%) were classified as moderate to severe (stage III-IV). Menstrual cycle stage (p=0.884) and ethnicity (0.288) were not different between cases and controls.

Table V. Demographic variables of the study population.

	CONTROLS (N=9)	**CASES (N=38)**	***P* VALUE**
Age, mean (±SD)	34.7 (7.95)	33.9 (4.25)	0.617
Lesion Type: (Frequency)*			
- **Endometrioma**	-	15(39)	N/A
- **Peritoneal Lesion**	-	11 (29)	
- **Deep- Infiltrating lesion**	-	13 (34)	
Stage, n(%):			
- **Stage 1 (Minimal)**	-	3 (8)	N/A
- **Stage 2 (Mild)**	-	3 (8)	
- **Stage 3 (Moderate)**	-	8 (21)	
- **Stage 4 (Severe)**	-	24 (63)	
Cycle phase at biopsy, n(%):			
- **Proliferative**	1 (11)	8 (21)	0.884
- **Menstrual**	3 (33)	9 (24)	
- **Secretory**	2 (22)	9 (24)	
- **Unknown**	3 (33)	12 (32)	
Age of first menses, y median (25%-75%)	13 (12.5-13.5)	12 (11-13)	0.436
Duration of bleeding, d median (25%-75%)	6 (4-7)	6 (4-8)	0.830
Ethnicity, n(%):			
- **Caucasian**	5 (56)	23 (61)	0.288
- **Asian**	3 (33)	5 (13)	
- **Unknown**	1 (11)	10 (26)	

3.2 miRNA Expression

3.2.1 Control vs eutopic endometrium of cases

Differences in the eutopic endometrium of women who develop endometriosis compared to those who do not has been suggested as a potential explanation for the disparity in the percentage of women with retrograde menstruation and the prevalence of endometriosis. Therefore, I compared miRNA expression in the eutopic endometrium of women with endometriosis and symptomatic controls. Specifically, miRNA expression was compared between control eutopic endometrium (n=9) and eutopic endometrium of women with endometriosis (n=38). miR-204 expression was significantly (p=0.016) down-regulated compared to symptomatic controls (***Fig. 3***) whereas there was no difference in miR-21, miR-424, and miR-10b (***Fig. 3***), miR-9 and miR-10a expression (***Fig. 4***).

3.2.2 Comparisons between ectopic implants

It is possible that identifying a diagnostic marker has been difficult because of the approach most researchers take in treating endometriosis as a homogenous disease. With its three major lesion types originating from different processes, it is likely these implants are biochemically different. Measuring differential miRNA expression in these implants would confirm they are different, and will also provide a basis for approaching endometriosis as a heterogeneous disease in near studies.

Expression of miR-21, miR-424, and miR-10b was differentially regulated between different lesion types. miR-21 expression was significantly lower in PE compared to OMA (p=0.004; ***Fig. 3***). A similar pattern of expression was observed with

miR-424, with significant down-regulation in PL compared to OMA (p=0.011; ***Fig.3***). miR-10b expression in DIE lesions was significantly lower than OMA (p=0.037; ***Fig. 3***). While miR-10a was not differentially expressed between different lesion types (p=0.122), linear regression analysis suggests a significant trend towards increased expression OMA vs. PE and DIE (p=0.049; ***Fig. 4***). Finally, there is no relationship between the expression of miR-9 (p=0.146) or miR-204 (p=0.131) with lesion type, however, linear regression analysis suggests a trend towards a relationship of miR-9 expression and ectopic implant (p=0.055, ***Fig. 4***).

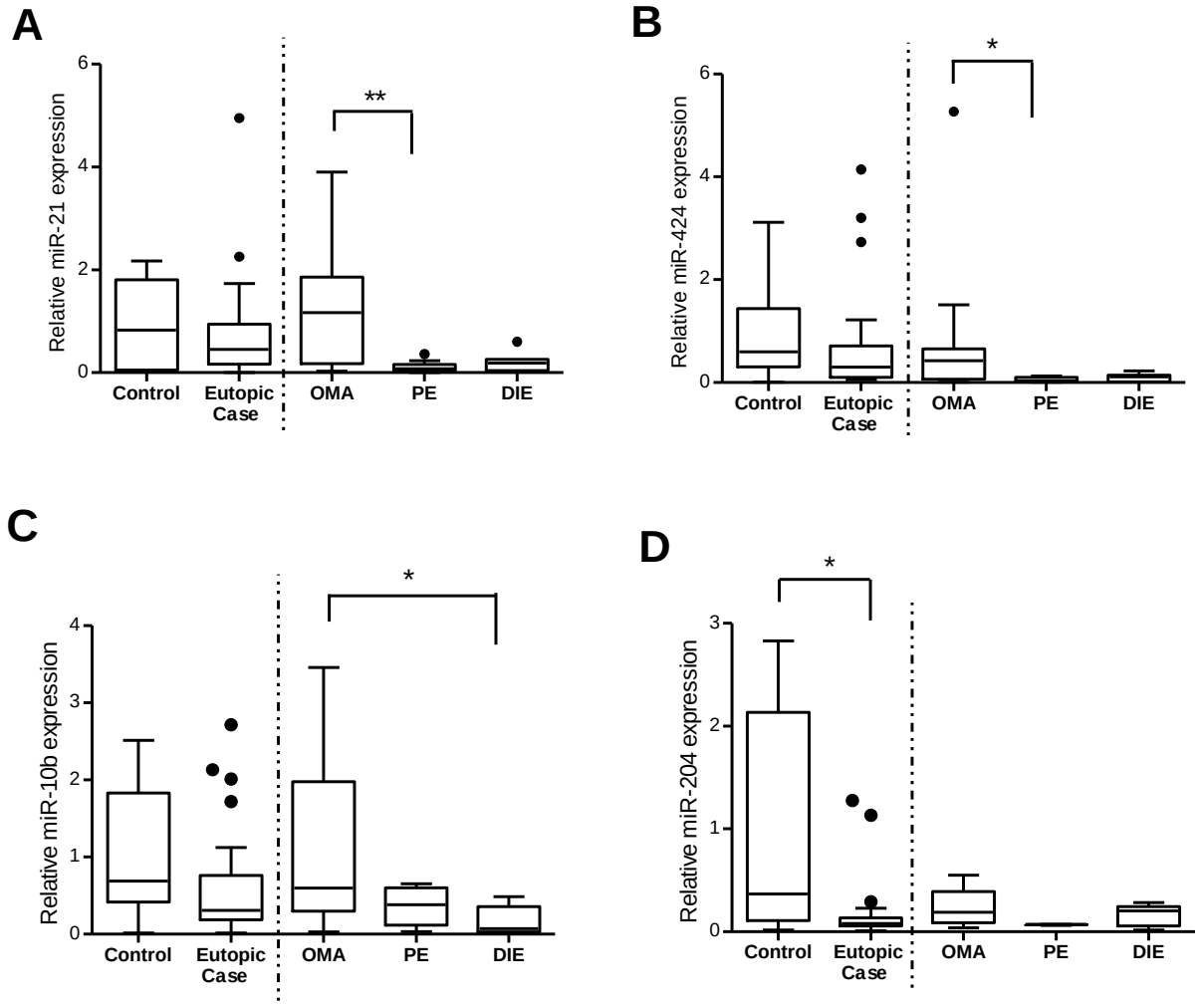


Figure 3. Relative microRNA expression in control endometrium (n=9) compared to eutopic endometrium (n=35), and microRNA expression between endometriotic implants. OMA (n=15), PE (n=11), DIE (n=10). The expression of (A) miR-21, (B) miR-424, (C) miR-10b, and (D) miR-204 normalized to reference genes SNORD68, SNORD95, and SNORD96A. *$p<0.05$, **$p<0.01$.

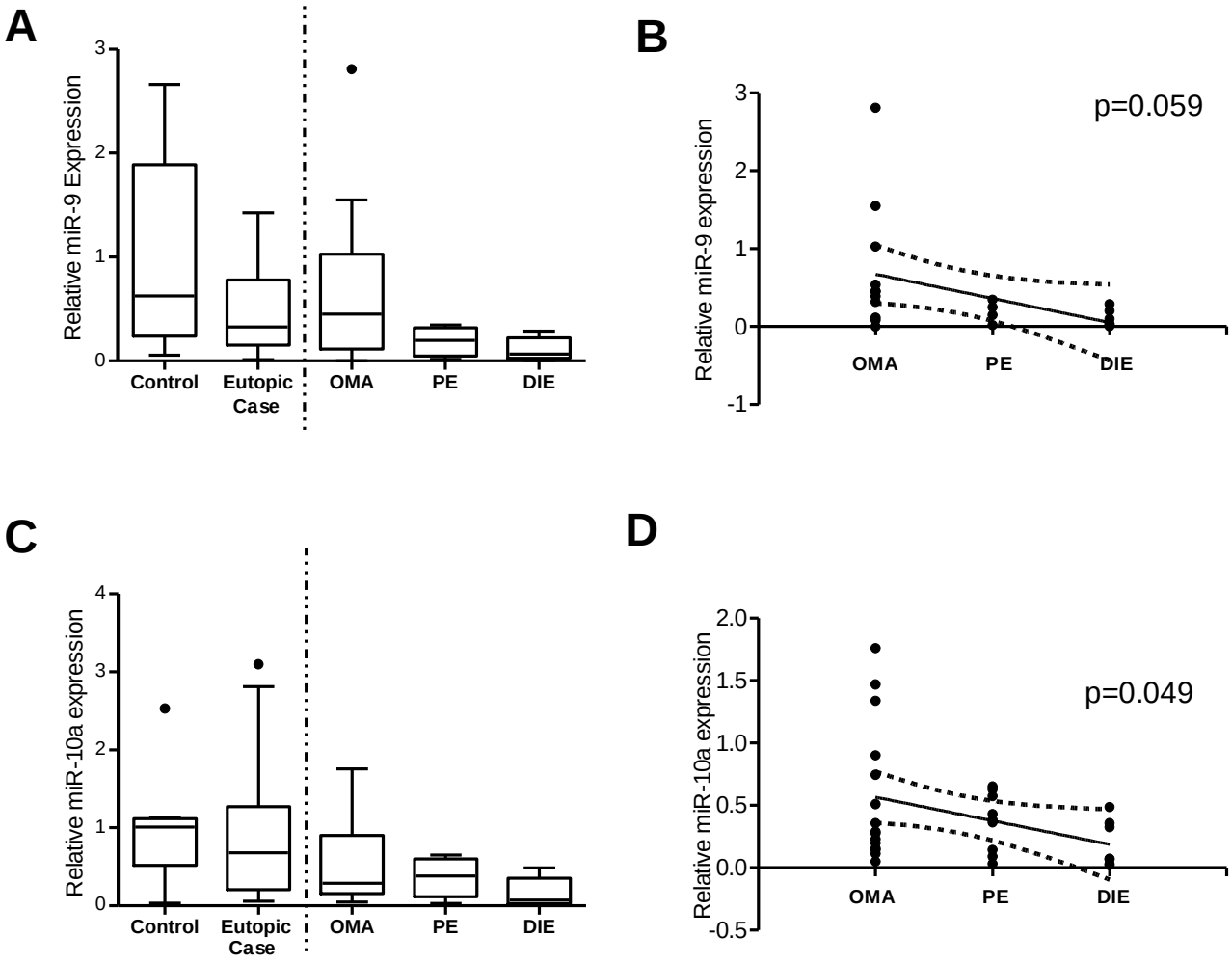
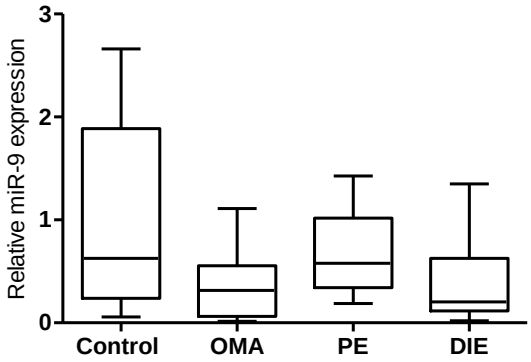
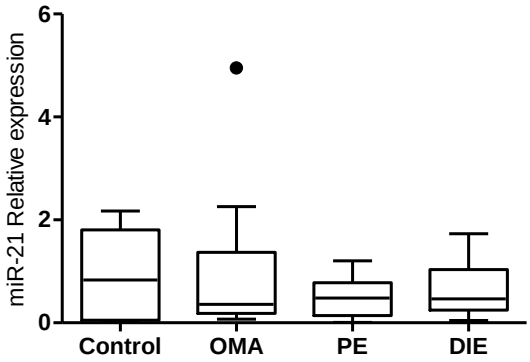


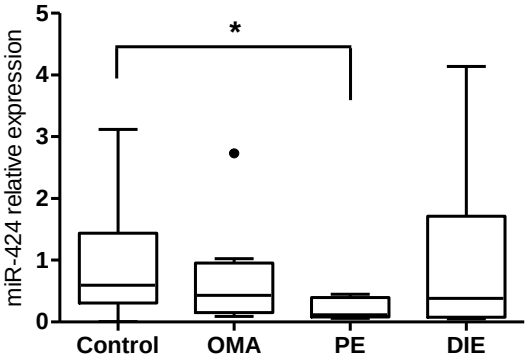
Figure 4. Relative microRNA expression in control endometrium (n=9) compared to eutopic endometrium (n=35), and microRNA expression between endometriotic implants. OMA (n=15), PE (n=11), DIE (n=10). The expression of (A) miR-9, with linear regression depicting the relationship of miR-9 and endometriotic implants (B). Expression of (C) miR-10a, and accompanying linear regression analysis between endometriotic implants (D). Data is normalized to reference genes SNORD68, SNORD95, and SNORD96A. Significance denoted $p<0.05$.

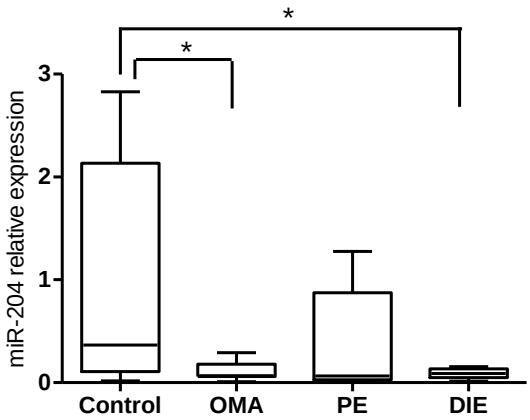
3.2.3 Control vs eutopic case endometrium by lesion type

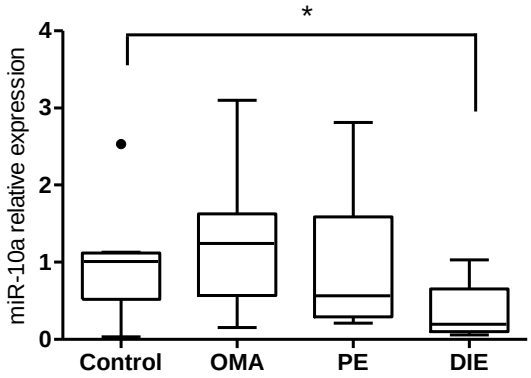
It has been proposed that the 10% of women who experience retrograde menstruation have endometriosis because of differences in their eutopic endometrium. Measuring differences in miRNA expression in the eutopic endometrium of cases and controls can help clarify what makes cases more susceptible to disease. miRNA expression varied greatly between eutopic endometrium of women with different lesion types, therefore I decided to investigate miRNA expression between the eutopic endometrium of women with specific lesion types. Expression of miR-424 is significantly lower in the eutopic endometrium of women with PE (n=11), compared to control endometrium (p=0.024; ***Fig. 5***). The eutopic endometrium of women with both OMA (n=15) and DIE (n=13) demonstrated lowered expression of miR-204 compared to controls with p=0.034, and p=0.027, respectively (***Fig. 5***). Finally, miR-10a and miR-10b demonstrate a similar pattern of expression in the eutopic endometrium of women with DIE compared to controls. miR-10a (p=0.028), and miR-10b expression was significantly lower (p=0.005) in cases compared to controls (***Fig. 5***).


Relative miR-9 expression
3
2
1
0
Control
OMA
PE
DIE


miR-21 Relative expression
6
4
2
0
Control
OMA
PE
DIE


miR-424 relative expression
*
5
4
3
2
1
0
Control
OMA
PE
DIE


miR-204 relative expression
*
*
3
2
1
0
Control
OMA
PE
DIE


miR-10a relative expression
*
4
3
2
1
0
Control
OMA
PE
DIE

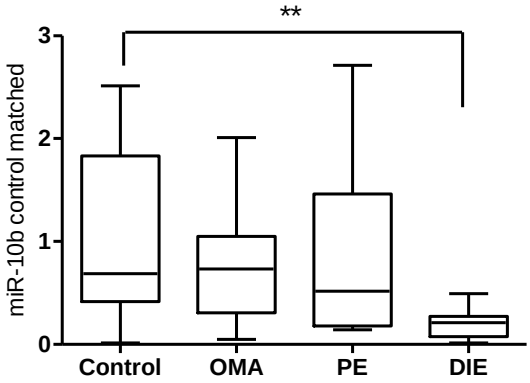

miR-10b control matched
**
3
2
1
0
Control
OMA
PE
DIE

Figure 5. MicroRNA expression between control endometrium (n=9) and eutopic endometrium of women with different lesion types. OMA (n=13), PE (n=10), DIE (n=12) Relative expression of (A) miR-9, (B) miR-21, (C) miR-424, (D) miR-204, (E) miR-10a, and (F) miR-10b. All data is normalized to reference genes SNORD48, SNORD95, and SNORD96A. *$p<0.05$, **$p<0.01$.

3.2.4 Ectopic lesion vs matched eutopic endometrium

Measuring miRNA expression in ectopic and matched eutopic endometrium may provide information about the biochemical differences between normal and diseased tissue. Because the participants in the current study had only one type of lesion, I was able to make the comparisons.

Expression of the following miRNAs is significantly lower compared to the matched eutopic endometrium. In women with PE, miR-9 (p=0.028), miR-21 (p=0.018), and miR-424 (p=0.011) were differentially expressed between these tissue types (***Fig. 6***). In women with DIE, the expression of miR-21 (p=0.031), and miR-424 (p=0.025) was lower in ectopic implants compared to matched eutopic endometrium. Finally, in women with OMA, lower expression of miR-10a (p=0.016) was observed in ectopic lesions compared to eutopic endometrium of the same women (***Fig. 6***). No change in miR-10b was noted when comparing each implant to its matched eutopic endometrium (***Fig. 6***).

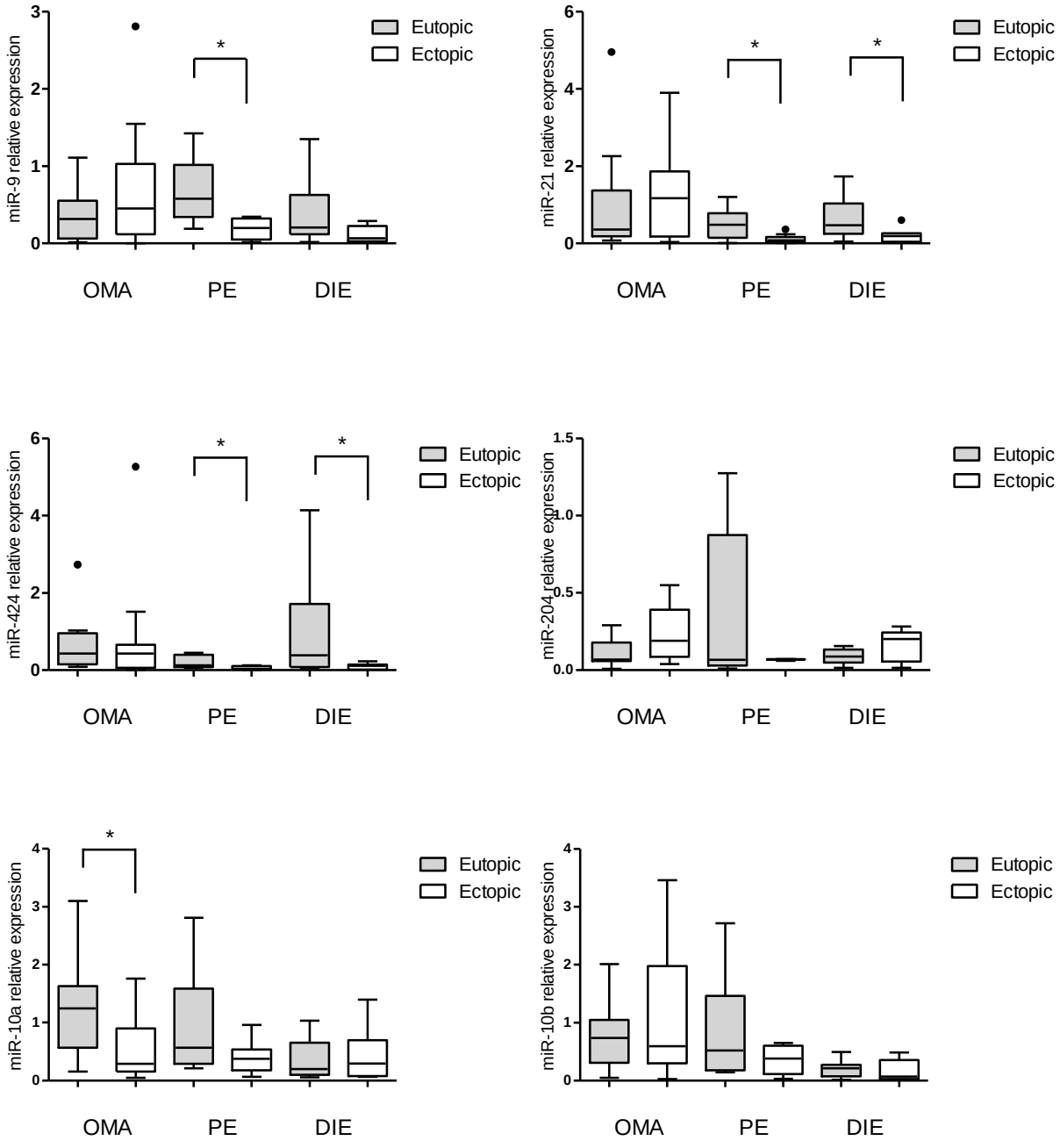

miR-9 relative expression
miR-21 relative expression
miR-424 relative expression
miR-204 relative expression
miR-10a relative expression
miR-10b relative expression
Eutopic
Ectopic
OMA
PE
DIE
*

Figure 6. MicroRNA expression in ectopic implants (OMA n=15, PE n=11, DIE n=10) compared to matched eutopic endometrium (OMA n=13, PE n=10, DIE n=12). MicroRNA expression of (A) miR-9, (B) miR-21, (C) miR-424, (D) miR-204, (E) miR-10a, and (F) miR-10b. All data is normalized to reference genes SNORD48, SNORD95, and SNORD96A. *$p<0.05$. The boxes in gray depict eutopic endometrium, and the white boxes represent ectopic implants.

3.3 miRNA and BDNF gene expression

3.3.1 Control vs eutopic endometrium of cases

Differences in miRNA expression warranted further investigation into the expression of known targets. In doing so, potential pathways involved in endometriosis development can be elucidated. BDNF gene expression was measured using R-qPCR with GAPDH as a reference gene. Data were not normally distributed, therefore a Mann-Whitney U Test was used to compare medians. No difference in BDNF gene expression was detected between endometrium from controls and endometrium of all the cases grouped together (p=0.470, ***Fig. 7)***.

3.3.2 Comparisons between ectopic implants

Using a Kruskal-Wallis analysis on ranks, the medians of BDNF gene expression were compared between OMAs, PEs, and DIEs. There was a statistically significant difference between the three lesion types (p=0.0001, ***Fig. 7***). Further pairwise comparison using Dunn's Method confirmed that the differences in BDNF expression were much higher in DIE compared to both OMA and PE. BDNF expression between OMA and PE was not different.

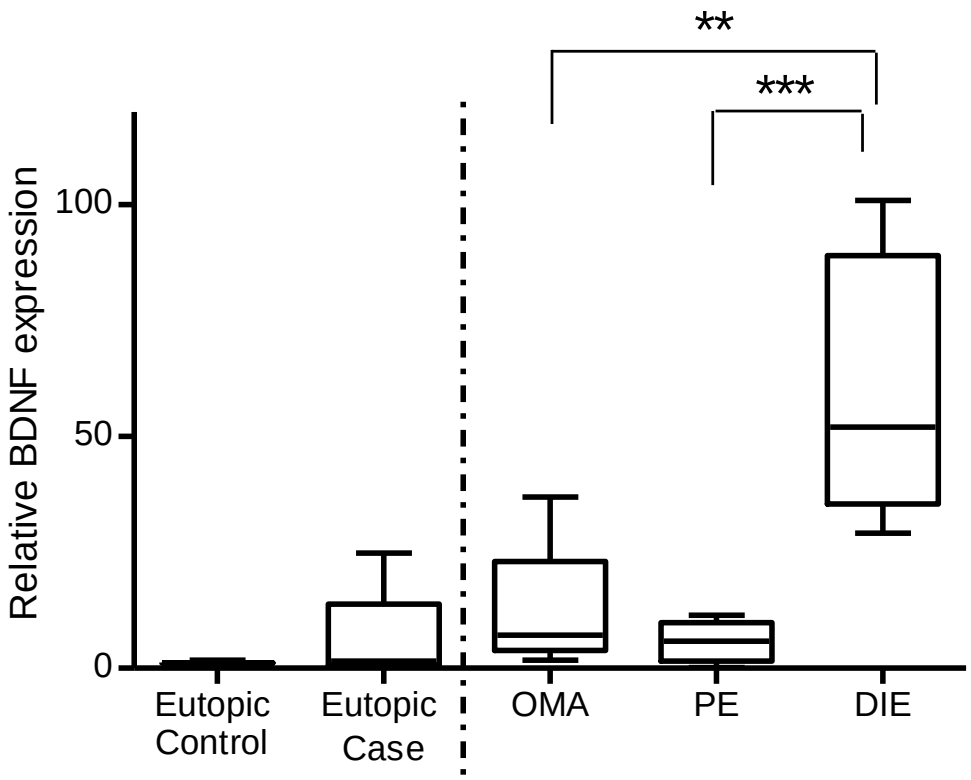


Figure 7. BDNF expression measured by RT-qPCR. On the left side of the graph is the comparison of control endometrium (n=3) to eutopic case endometrium (n=24) of women with endometriosis. On the right side, BDNF expression in the three ectopic implants are compared. OMA (n=12), PE (n=9), DIE (n=9) All data is normalized to GAPDH.**$p<0.01$, ***$p<0.001$.

3.3.3 Control vs eutopic case endometrium by lesion type

Data were not normally distributed, therefore a Kruskal-Wallis analysis on ranks was used to compare median BDNF expression. BDNF expression was compared between endometrium of symptomatic controls and matched eutopic endometrium of women with different lesion types. There was a statistically significant difference in BDNF expression between these tissue types (p=0.003, ***Fig. 8***). Using Dunn's method for pairwise comparison, it was determined that BDNF expression in the eutopic endometrium of women with DIE is much higher than women with OMA and PE. There was no difference in the eutopic endometrium of controls and any case endometrium, and no differences between eutopic endometrium of women with OMA and PE.

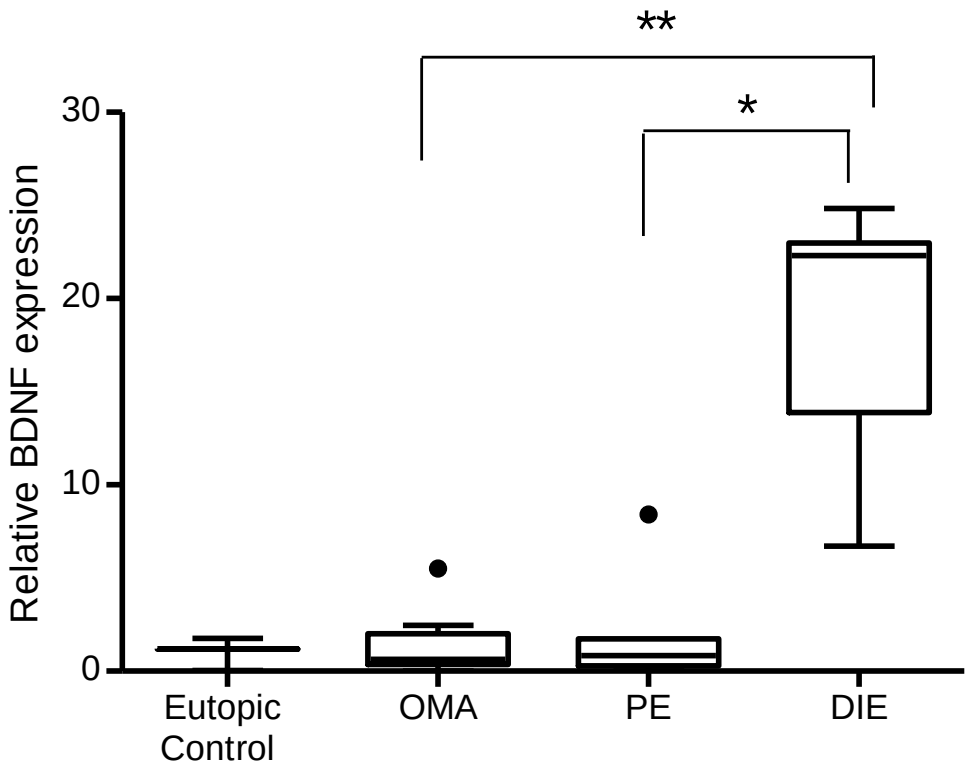


Figure 8. BDNF expression in eutopic endometrium of symptomatic controls (n=3) compared to the eutopic endometrium of women with ovarian endometrioma (OMA, n=9), peritoneal lesions (PE, n=8) and deeply-infiltrating endometriosis (DIE, n=7). All Data is normalized to GAPDH. *p<0.05, **p<0.01

3.3.4 Ectopic lesions vs matched eutopic endometrium

Data were not normally distributed therefore a Mann-Whitney U Test was used to measure BDNF expression of ectopic lesions to the eutopic endometrium of the same women. Between OMA and eutopic endometrium, there was as significant increase in BDNF expression (p=0.0007, ***Fig. 9***). In DIE, the same pattern was observed, with ectopic lesion expressing BDNF much more (p=0.0002). Ectopic and eutopic endometrium of women with PE did not show changes in BDNF expression (p=0.094).

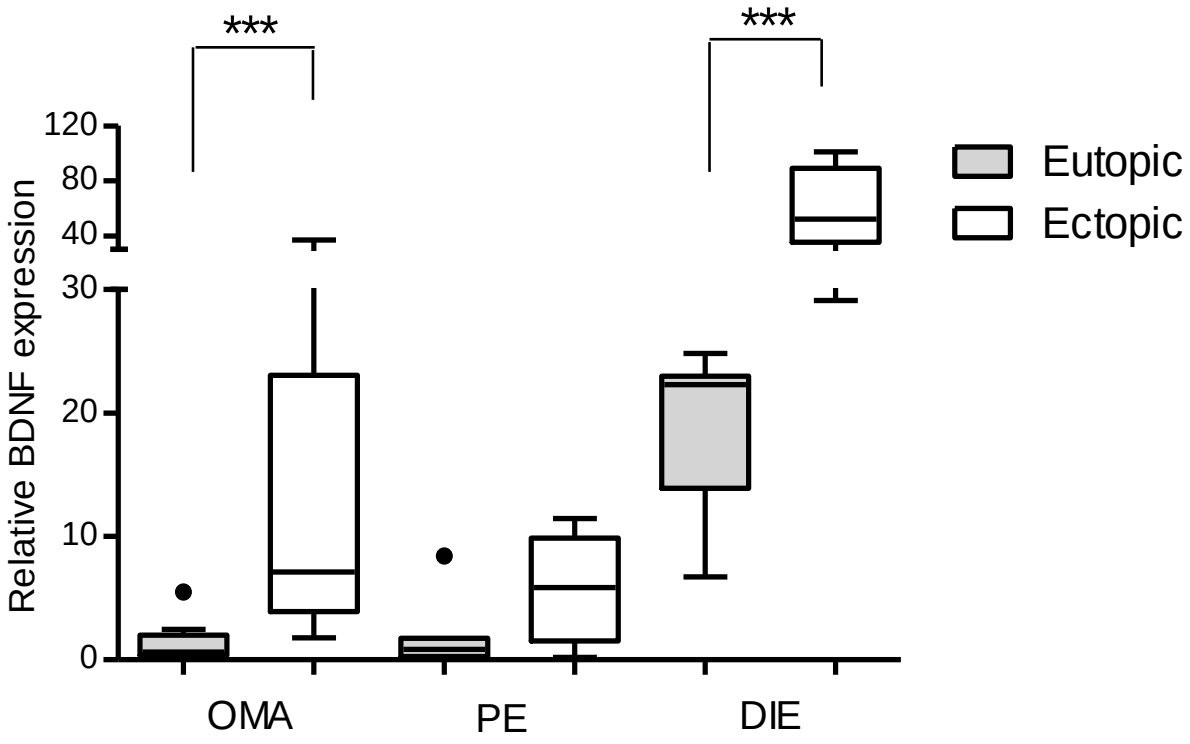


Figure 9. BDNF expression in ectopic lesions (OMA n=12, PE n=9, DIE n=9) compared to matched eutopic endometrium (OMA n=9, PE n=8, DIE n=7). All data are normalized to GAPDH. ***p<0.001.

3.4 BDNF protein expression

After identifying changes in BDNF gene expression in endometriotic lesions compared to eutopic endometrium, I used western blot analysis to identify differences in protein expression was present. Western blot analysis revealed a ~32 kDa band, indicating a BDNF precursor. The mature form at 15 kDa was not detected. There were no changes observed in BDNF protein expression between control endometrium and eutopic endometrium, or between ectopic lesions (***Fig. 10***). Using a student's t-test, BDNF protein expression was unchanged between the eutopic endometrium of controls and cases ($p=0.681$), and using a One-way ANOVA, BDNF protein expression was not different between OMA, PE, and DIE ($p=0.275$). Furthermore, the linear regression analysis on implants did not detect any relationship between BDNF protein and implant type ($p=0.140$, ***Fig. 11***). BDNF expression was also unchanged between OMA and matched eutopic endometrium ($p=0.589$), between PE and matched eutopic endometrium ($p=0.792$), and between DIE and matched eutopic endometrium ($p=0.189$, ***Fig.12***).

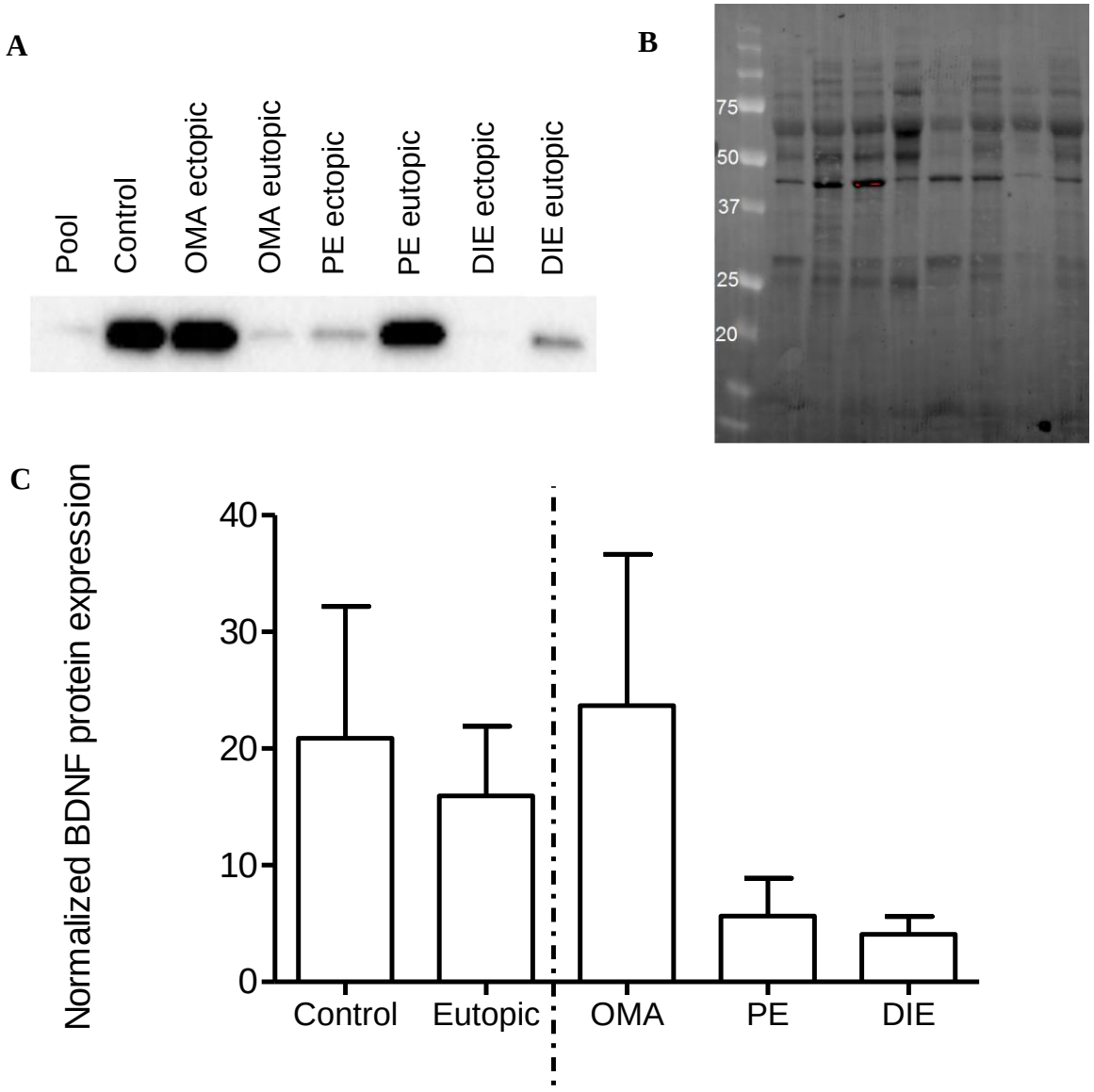


Figure 10. BDNF protein expression. **A.** Immunoblot image of BDNF protein (~28 kDa) in different tissue types, bands were normalized using total lane normalization with stain-free technology. **B.** Stain free image of total lane protein. **C.** On the left side, BDNF expression is compared between control endometrium (n=6) and eutopic endometrium from cases (n=16). On the right side, BDNF expression is compared between the three ectopic implants: ovarian endometrioma (OMA, n=6), peritoneal lesions (PE, n=5), and deeply-infiltrating endometriosis (DIE, n=4).

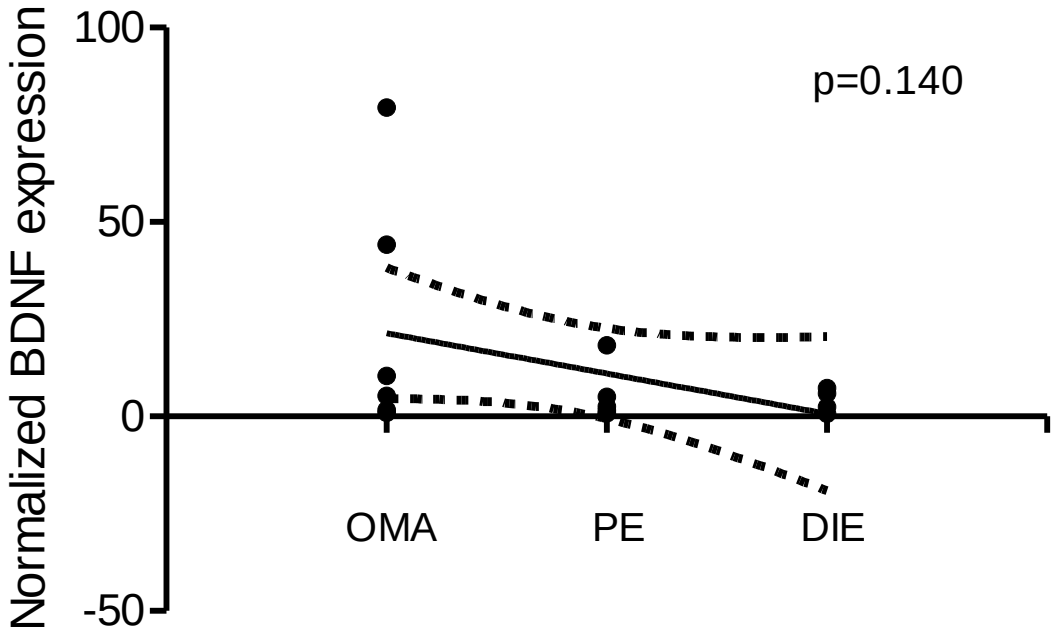


Figure 11. Linear regression analysis on BDNF protein expression between different endometriotic lesions. OMA (n=6); PE (n=5); and DIE (n=4).

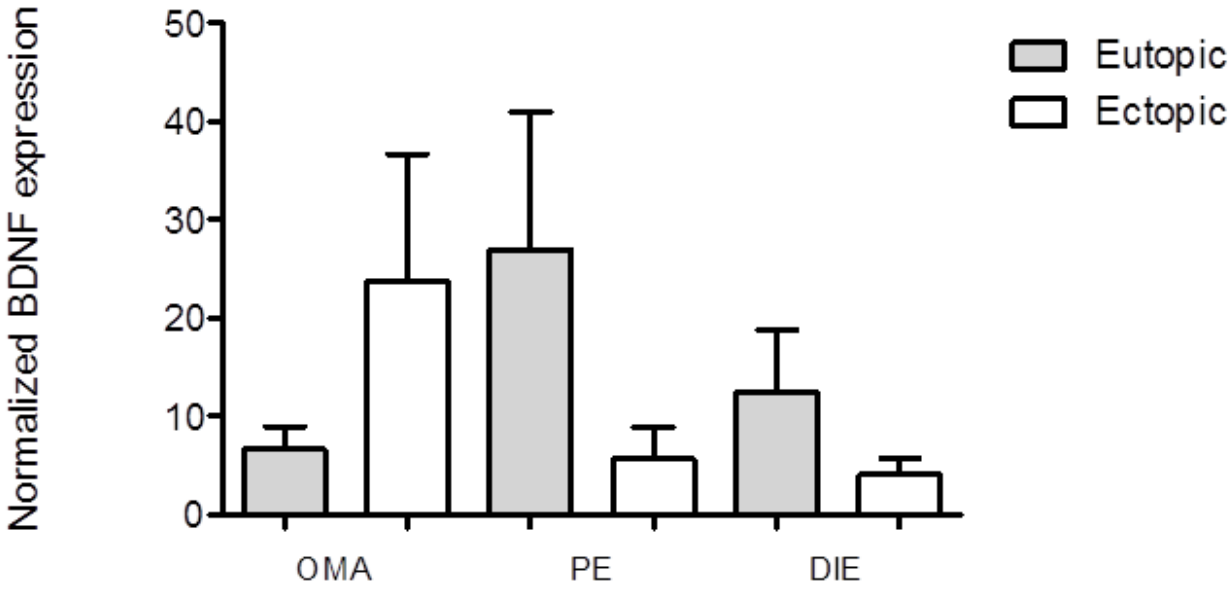


Figure 12. Normalized BDNF protein expression in ectopic lesions (OMA n=6; PE n=5; DIE n=4) compared to the matched eutopic endometrium (OMA n=6; PE n=6; DIE n=4).

3.5 Plasma BDNF

Past studies have identified upregulated plasma BDNF in women with endometriosis. Since miRNAs that regulate BDNF are dysregulated in my samples, I decided to measure plasma BDNF as well. There were no significant differences in plasma BDNF between controls and women with endometriosis (p=0.341, ***Fig. 13***). One-Way ANOVA did not detect any significant changes between controls and implant types (p=0.285). Furthermore, t-tests did not detect differences in plasma BDNF between controls and OMAs (p=0.210), PEs (p=0.129), and DIEs (p=0.976). Finally, there were no changes in plasma BDNF when comparing women at different stages of disease (p=0.620).

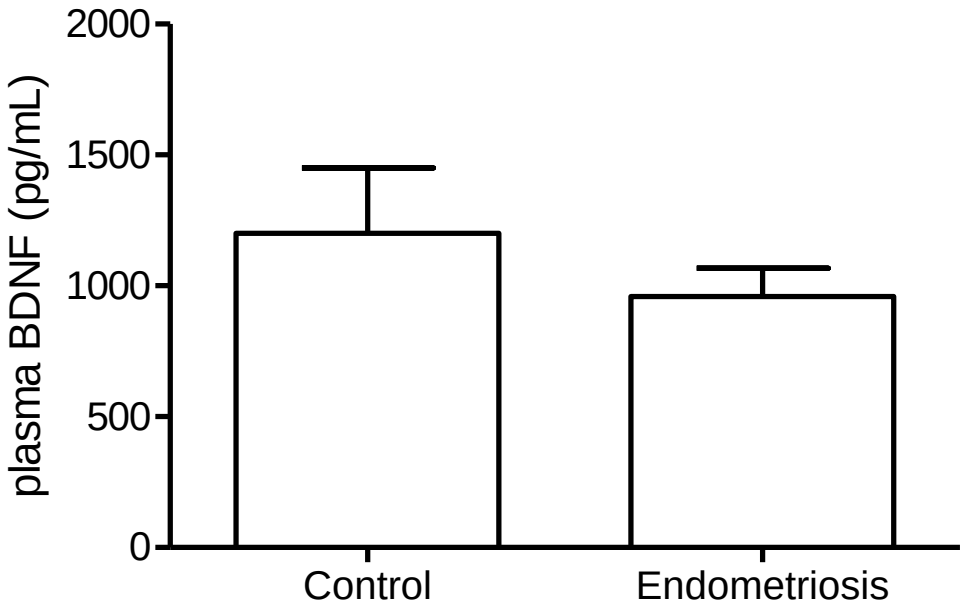


Figure 13. Plasma BDNF between symptomatic controls (n=9) and women with endometriosis (n=37).

CHAPTER FOUR: DISCUSSION

4.1 Summary of Findings

Endometriosis is a complex estrogen dependent disease of unknown cause lacking a simple diagnostic test for which therapeutic options remain suboptimal. Advances in genomic, proteomic and metabolomic techniques have identified novel miRNAs, genes, and proteins that are differentially expressed in women with endometriosis compared to control populations. However, identification of specific markers with suitable sensitivity and specificity that correlate with clinical outcomes and provide insight for prognosis remains elusive. In the current study, miRNA expression was measured in women with endometriosis (with different lesion types), and symptomatic women without endometriosis. miRNAs were selected on the basis of their prevalence in the current literature, and association with BDNF. miRNA expression was measured between control and eutopic case endometrium, between ectopic implants and matched eutopic controls, and between ectopic lesion types.

My results demonstrate that miR-204 was the only miR downregulated in the eutopic endometrium of women with endometriosis compared to controls. However, I have also shown that when controlling for lesion type, there is a significant difference in the expression of all miRs studied in the eutopic endometrium of cases compared to controls. miRNA expression was also shown to be different between lesion types. For example, while miR-424 was down-regulated in PE compared to OMA, no differences were observed in DIE. On the other hand, miR-10b expression was significantly lower in

DIE compared to OMAs. This indicates that OMAs, PE and DIE lesions are biochemically distinct entities and should be treated as such in future miRNA studies.

As well as identifying differential miRNA expression in endometriotic lesions, miRNAs that regulate BDNF were differently regulated. Specifically, miR-204 was downregulated in eutopic endometrium compared to controls, a finding consistent with reports of increased BDNF gene and protein expression in the eutopic endometrium of women with endometriosis vs controls[69,139]. miR-10b was differentially regulated in DIE compared to OMAs, and miR-10a and miR-10b were downregulated in the eutopic endometrium of women with DIE compared to control endometrium. A relationship between miR-10a expression and lesion type was also confirmed, further supporting my hypothesis that expression of miRNA associated with BDNF are lower in cases vs controls.

The expression of BDNF mRNA in these lesion types was significantly elevated in eutopic and ectopic tissue of women with DIE compared to the ectopic and eutopic tissue of women with OMA and PE. Furthermore, BDNF mRNA in OMAs and DIE were elevated compared to their eutopic tissue counterparts. Although BDNF gene expression was increased, BDNF protein expression was unchanged between controls and eutopic endometrium, and between lesion types. Finally, plasma BDNF was unchanged between women with endometriosis and controls, even after controlling for stage and lesion type.

4.2 MicroRNA expression

In the current study, I found that miR-424 expression was significantly upregulated in OMAs compared to PE sites by 14.5-fold, downregulated in the eutopic endometrium of women with PE compared to control endometrium by 4.5-fold, and downregulated in ectopic sites PE and DIE lesions compared to their matched eutopic sites by 4.4- and 10.6-fold respectively. These results contrast with those of a previous study[60] in which miR-424 expression was higher in the eutopic endometrium from women without endometriosis compared to women with endometriosis by 3.4-fold[60]. Furthermore, expression of miR-424 was significantly upregulated in OMAs compared to eutopic case endometrium by 1.5-fold, and upregulated in PE and DIE compared to both eutopic case endometrium (2.5- and 2.7-fold, respectively) and control endometrium (1.8- and 1.9-fold respectively)[60]. Divergent results may be explained by differences in the criteria used to assign participants to the control groups. In the current study, women in the control groups were undergoing laparoscopy for pelvic pain and/or infertility, whereas in the prior study[60], the control group consisted of healthy women undergoing laparoscopic tubal sterilization. I suggest that the more appropriate comparison should involve symptomatic women. In endometrial tissue of women with endometriosis, miR-424 negatively regulated vascular endothelial growth factor (VEGF) expression[60]. Furthermore, miR-424 was found to target VEGF and basic fibroblast growth factor (bFGF) via VEGF receptor 2 and FGF receptor 1, respectively, in endothelial cells, reducing cell proliferation and migration in vitro[73]. Angiogenesis is an important process in endometriosis, as the establishment of a blood supply to the lesion is necessary for its

growth and survival. However, it is important to note that while some studies documented differences in VEGF[57,60,108], others have been unable to do so[140–142].

Although endometriosis is a benign disease, the lesions share many of the characteristics of cancer, such as angiogenesis and resistance to apoptosis. In endometrial cancer cells, overexpression of miR-424 suppressed 17β-estradiol-induced cell proliferation by targeting the PI3K/AKT signaling pathway mediated by G-protein coupled receptor[143]. Therefore I postulate that lower expression of miR-424 in ectopic tissues of the current study contributes to over expression of VEGF and increased estradiol-induced cell proliferation of endometriotic lesions.

Similar to the pattern of miR-424, miR-21 expression was increased in OMAs compared to PE. Expression was also higher in eutopic endometrium of women with PE and DIE, compared to the respective lesion type. No changes in the eutopic endometrium of cases with any lesion type, compared to controls, were observed. These results also conflict with those of a prior study[108], with increased miR-21 expression in OMAs compared to eutopic endometrium of cases by 1.9-fold, elevated expression in OMAs and DIE compared to control endometrium (1.7- and 2.2-fold, respectively), and no changes observed in PE. I suggest that divergent findings can be explained by differences in comparisons made and the burden of disease in study participants. Specifically, in the present study, I did not compare miRNA expression between ectopic lesions and eutopic endometrium of controls. We are unsure of the biological relevance of comparisons between ectopic lesions in women with endometriosis and eutopic endometrium of asymptomatic women without endometriosis. In the present study, I limited comparisons

to eutopic endometrium and ectopic lesions within cases, assuming that differential expression of the miRNAs within the women affected would reflect disease processes as opposed to individual specific differences in gene expression and regulation. Moreover, in the current study, I was careful to choose specimens from our tissue bank from women with solely one lesion type (except one case) to avoid potential influences of disease burden on miRNA expression. In the previous study[108], the DIE and PE groups were subsets of the OMA group, containing women with 2 or more lesion types.

Although miR-21 expression has been documented in women with endometriosis[108,122], its role in endometriosis is unknown. Overexpression of miR-21 has been identified in a number of cancers, and miR-21 targets Phosphatase and Tensin homolog (PTEN), a tumour suppressor, Programmed Cell Death protein 4 and B-cell lymphoma 2 (Bcl-2), a known cell death regulator [135,144]. Both PTEN and Bcl-2 expression are dysregulated in endometriosis[145–149]. Furthermore, miR-21 upregulation inhibits RECK (also known as reversion-inducing-cysteine-rich protein with kazal motif), whose targets are MMPs-2 and -9 [150]. MMP-2 and -9 expression and enzyme activity are increased in women with endometriosis compared to controls[151,152]. In contrast, in endometrioid endometrial carcinoma (EEC) tissue and plasma samples, miR-21 was found to be significantly downregulated via next generation sequencing (NGS) analysis in tissue (6.3-fold) and via qPCR in plasma (1.5-fold), however no targets in miR-21 were discussed in this paper[82].

Results of my thesis research demonstrated that miR-9 expression was significantly downregulated in women with PE compared to the matched eutopic

endometrium by 3.6-fold. No change in miR-9 expression was found between ectopic lesion types or between eutopic endometrium of cases and controls. Only one other study has investigated miR-9 expression and its corresponding passenger strand (miR-9*) in the context of endometriosis[91]. In early secretory endometrium of women with (n=4) and without (n=3) endometriosis, microarray confirmation with qPCR determined miR-9 expression was downregulated in women with endometriosis (25.6-fold, $p<0.05$)[91]. Although there is agreement in the direction, the magnitude of miR-9 expression is more profound in this study[91]. This study did not include ectopic endometriotic lesions in their analysis. Pathway analysis confirmed that Bcl-2 mRNA was a miR-9 target[91] and consistent with the pro-survival phenotype observed in endometriotic cells. Dysregulation of miR-9 is also associated with many types of cancers[153,154]. In ovarian cancer specifically, the suppression of miR-9 results has been shown to increase cell proliferation, cell growth, and anti-apoptotic properties *in vitro*[153]. These effects are mediated by binding to the 3' untranslated region (UTR) of nuclear factor κB (NF-κB), which then promotes oncogenic processes such as cell proliferation, cell adhesion and angiogenesis through molecules such as cyclin D1 (CCND1), matrix metalloproteinases and VEGF, respectively[153,155,156].

It has previously been shown that circulating concentrations of BDNF are elevated in women with endometriosis compared to healthy control populations[69,94,95]. Prior studies in ovarian granulosa cells have shown that miR-10a, -10b and -204 regulate BDNF expression[157–159] and thus I chose these miRs for examination in the current study. My results revealed that miR-10a is downregulated in the eutopic endometrium of women

with DIE compared to controls by 2.8-fold, and in OMAs it is downregulated in ectopic vs eutopic tissue by 2.2-fold. Linear regression indicates a relationship between lesion type and miR-10a expression (p=0.049). miR-10b, which shares the same seed sequence as miR-10a and therefore share the same group of targets, is also downregulated in the eutopic endometrium of women with DIE compared to controls (5.1-fold), and differentially expressed across lesion types, specifically it was significantly upregulated in OMAs compared to DIE (5.8-fold). Finally, miR-204 was significantly downregulated by 6.1-fold in the eutopic endometrium of women with endometriosis compared to control endometrium. Specifically, the eutopic endometrium of women with OMAs and DIE expresses miR-204 much less than controls, by 9.2- and 11.5-fold, respectively. Taken together, my results suggest that miRNAs previously shown to regulate BDNF expression in other target tissues are dysregulated in women with endometriosis.

One study, using NGS analysis, identified that miR-10a was downregulated in endometriomas compared to the endometrium of symptomatic controls (magnitude not indicated)[128]. In human endometriotic cells, miR-10b has also been found to target and inversely regulate syndecan-1 (SDC-1) in endometriosis[160]. SDC1 is a transmembrane heparin sulfate proteoglycan involved in many processes in endometriosis. In human endometriotic cells, increased miR-10b resulted in decreased SDC-1 mediated cell invasiveness via downregulation of MMPs and protease inhibitor PAI-1, which are normally upregulated in endometriosis[58,160]. Using an *in silico* approach, another group discovered that miR-10b negatively regulates the expression of BDNF by binding to its 3' UTR region[158]. Furthermore, miR-10b directly targets BDNF in goat granulosa cells,

inhibiting cell proliferation[157]. Reintroduction of BDNF to the cells reversed the suppressive action of miR-10b, promoting cell growth and proliferation. In epithelial ovarian cancer (EOC) cell lines HO-8910 and SK-OV-3, changes in miR-204 expression was associated with changes in apoptosis sensitivity[159]. Upregulation of miR-204 led to decreased resistance to apoptosis in these cells, as well as decreasing cell invasion and BDNF expression.

4.3 BDNF gene expression

After confirming that miRNAs shown to regulate BDNF are differentially regulated in women with endometriosis, the next logical step was to measure the gene expression of BDNF in these same tissues. In the current study, BDNF gene expression was significantly elevated in DIE compared to both OMA (13.6-fold) and PE (10.4-fold), as well as in the eutopic endometrium of women with DIE compared to that of women with OMA (4.6-fold) and PE (10.1-fold). Furthermore, OMA and DIE were elevated in ectopic tissue compared to matched eutopic endometrium (8.8- and 3.2-fold, respectively). The downregulation of miR-10a, -10b, and -204 coincides with the upregulation of BDNF in these tissues. These results are consistent with the notion that miRNAs negatively regulate their mRNA targets.

In the present study, changes in BDNF protein expression in the tissues samples and plasma could not be demonstrated. The immunoblots showed that the predominant BDNF species detected were ~28-kD precursor isoforms, with only trace amounts of the mature, processed peptides of 14-15 kDa in some samples (data not shown). I speculate that is possible that the biopsies reflected a pool of the BDNF precursor, rather than the

processed, secreted products. Mature BDNF is likely to be secreted from cells, however, with overexpression, it is possible that BDNF precursors can be secreted to the extracellular milieu[93,161]. This was also observed in a previous study[93], and the authors suggested that this finding may have important implications, since interference of BDNF processing in the endometrium can represent a novel therapeutic strategy to reduce mature BDNF production.

BDNF protein levels have previously been shown to be higher in endometriosis compared to controls[93]. Relative abundance of mRNA and proteins are not always well correlated[162]. Protein abundance regulation mirrors specific biological roles: for example a regulatory protein would be produced and degraded a higher rates as it reacts to stimuli, but structural proteins would last much longer[162]. There has been evidence of an underappreciated role for post-transcriptional, translational, and degradation regulation in the determination of protein concentrations. Of course, it is possible that there is a simpler explanation to these results. In my thesis only 4-6 tissue samples for each group were examined, and thus this analysis is under-powered. Few samples were used due to the limited availability of samples from women with only one lesion type.

There was also no change in plasma BDNF between women with and without endometriosis, which is contrary to results from previous studies[69,94,95]. Subanalysis using stage of endometriosis and plasma of women with specific lesion types also did not reveal differences in circulating BDNF expression. The current study population is a subset of the population used previously[69]. In addition, the number of women with early stage disease in the current study was rather small (n=6) and thus underpowered. I also note

that previous differences in plasma BDNF were identified in early stage disease[69,94]. Due to the many exclusion criteria in this study, the control population was not large (n=9). Furthermore, the study population does not represent the clinical population of women who would show up in the clinic for this test, given that smokers, certain medications, and comorbidities were excluded from this study, while previously[69] these exclusions were not made. Therefore, although results are different from prior studies, I do not propose that they invalidate the relationship observed previously.

The current study is the first to measure BDNF expression in all endometriotic lesion types. Previously, BDNF gene expression was found to be significantly upregulated in OMA compared to matched eutopic endometrium of women with endometriosis by 9.7-fold[163], but other ectopic sites were not investigated. Furthermore, the neurotrophic tyrosine receptor kinase 2 (Ntrk2), a specific BDNF receptor, was also upregulated in OMA compared to eutopic endometrium (8.7-fold) and harmonious with results of the current study with upregulation in OMA (8.8-fold). The expression of BDNF and Ntrk2 in the uterus is conserved across many species including humans, rats, mice, pigs and bats, suggesting they play an important role in the reproductive function of females[164]. In a previous study[165], daily exposure to estradiol in ovariectomized mice increased the expression of BDNF and its low affinity receptor nerve growth factor receptor (NGFR). Since endometriosis is estrogen-dependent, the increased estrogen levels influencing BDNF expression likely play a role in endometrial pathology.

Although the role of BDNF in reproductive physiology is not well understood[164], links have been made to the genesis of endometriotic nerve fibre networks, due to the

correlation of plasma BDNF and severity of pelvic pain in women with endometriosis[95]. A high degree of staining for BDNF in the glands and stroma of PE and DIE has suggested a role of BDNF in the innervation of endometriosis[166]. This result is consistent with my findings of increase BDNF gene expression in DIE lesions and OMA. These manifestations of endometriosis are considered more painful[48]. However, in another study pain scores and BDNF levels in tissue were not correlated[163]. Another potential role of BDNF in the endometrium is the resistance to apoptosis via binding to the full-length form of Ntrk2[94].

4.4 Replicability of study results

Replicability of miRNA results has been a challenge facing the field. The study of alterations in gene expression without inducing changes in the genetic code is termed epigenetics, and it has provided the most promising avenue to pursue. This is in part due to the discovery of microRNAs[167–169] and other post-transcriptional modifications[85,170,171]. Soon, the regulatory role of miRNAs and their implications in disease became apparent[172]. MicroRNAs in the study of endometriosis began in 2007 with an article (that has since been retracted) using microRNA profiling to identify differential expression of miRNA in ectopic and eutopic endometrium of women with endometriosis and controls[173]. Soon after, others followed suit and studies on differential microRNA expression has expanded to serum[46,104,174–176], plasma[90,106,107], and peritoneal fluid[177], as well as using panels of miRNAs[105].

Numerous studies have identified an array of miRNAs whose expression is dysregulated in women with endometriosis compared to a control population. While many exciting studies have been published, very few of the different miRNA identified as being differentially expressed have been reproduced in other studies. This begs the question: is the lack of reproducibility truly due to inability to replicate previous results, or absence of efforts aimed at trying to reproduce prior results? The majority of groups have used microarrays to catalogue miRNAs potentially dysregulated in women with endometriosis compared to control groups (**Tables IV and V**). Differences in results arising from studies quantifying targeted miRNA expression can occur due to the differences in study populations, different equipment, and protocols used. However, one may argue that these differences should not affect the result if the biomarker is robust. Few studies have produced the same results, such as the downregulation of miR-141 in serum of women with endometriosis[88,107], upregulation of miR-145 in ectopic implants compared to eutopic endometrium[87,123], upregulation of miR-29c in OMAs compared to controls[60,128], downregulation of miR-200b in OMAs[127,128], among a few others (**Tables IV and V**). Furthermore, some of the results from the current study have been shown previously. I have shown that miR-424 is downregulated in ectopic tissue compared to paired eutopic endometrium. Down-regulation of miR-424 was seen in ectopic tissues compared to paired eutopic endometrium, confirming results of another study[178]. I have also shown that miR-9 is downregulated in endometriosis compared to controls, and another group has done the same, however their study did not include ectopic lesions[91]. miR-10a, -10b, and -204 are all miRNAs that have not been measured in endometriotic

lesions. Seeing as these miRNA are dysregulated as well as BDNF gene expression, it may be an interesting avenue to pursue in future studies.

While some miRNAs that I have studied have shown similar expression to other studies[91,128], there are conflicts as well. miR-21 expression was upregulated in endometriosis in other studies, unlike what I have shown here[108,128]. Upregulation of miR-424 has also been shown in a previous study[60], which conflicts with what I have shown. As previously mentioned, it is not uncommon to have conflicting results in this field of study[88,91,103,127,130], but determining the reason behind the differences is important to understand the role miRNA have in endometriosis. I did not perform a microarray in the current study, and I believe doing so would not have added any value to the current literature, since identifying novel miRNAs is something that has been done. Attempting to reproduce results from other studies is more constructive.

While microarray studies do a great job at identifying novel miRNAs, I propose the focus should now be validating these results in larger populations of women, and identifying targets for diagnostic purposes and/or therapy.

4.5 Relevance of Findings

While the current literature has established that miRNA are dysregulated in endometriosis, not much progress has been made since in terms of identifying specific pathways involving miRNAs that lead to the pathogenesis of disease. These differences are undoubtedly, but not exclusively, attributed to differences in study design. The current study is one of four studies[60,108,129] measuring miRNA expression in all endometriotic

lesion types. Of these four studies, two are from the same laboratory, and have used the same study population[60,108]. This shows that the vast majority of studies do not take into account that lesion types present differently and can have an effect on the overall miRNA profile of an individual. Failure to measure miRNA expression in all endometriotic lesions is a failure to recognize endometriosis as a heterogeneous disease. In excluding certain lesion types, potentially valuable information about the pathways leading to endometriotic lesion formation is not collected.

Another drawback from current studies is the comparison groups that are used vary from study to study. While some may use paired eutopic endometrium as a control (some may even use it as a case sample), others will use control endometrium from healthy controls, or symptomatic controls, and even peritoneum that is adjacent to the ectopic lesions collected[130]. More comparison groups will provide insight into what pathways are dysregulated. Comparing endometrium of cases and controls can help to identify differences in pathogenesis of disease, specifically what makes women with endometriosis more likely to develop disease. While comparing the endometrium of cases to their respective ectopic lesions gives information about the particular lesion type, how and why it has developed.

Other study characteristics including study populations, normalization methods, statistical methods, sample handling, primers used, among other differences, can contribute to differences in miRNA expression between studies.

The current study adds valuable information to the miRNA literature, as it shows differential expression of miRNA between lesion types. This confirms that lesion types

are truly biochemically distinct. My results, together with other previous studies[60,73], identify a potential relationship between dysregulated miR-424 with angiogenic pathways. Furthermore, the dysregulation of miR-10a, -10b, and -204 in conjunction with dysregulated BDNF indicates a relationship between BDNF expression and endometriosis pathology, regulated by these miRNA. I have not identified through what pathways BDNF functions in endometriosis, but other data have suggested the role of BDNF in resistance to apoptosis[159], and pain pathways[163,166,179].

The miRNA results may not provide enough evidence for their use as a diagnostic marker as more validations are necessary, and tissue biopsies are not considered minimally invasive. However, these results in conjunction with other research can provide information on potential drug targets for treatments[52]. Because this study was performed on tissue, associating specific miRs with specific lesion types can also provide information to surgeons on what types of lesions they expect to see during surgeries. Furthermore, miRNA expression may be able to provide information on burden of disease.

4.6 Strengths and Limitations

Strengths of this study include the use of symptomatic controls with surgical exclusion of endometriosis. Women with endometriosis were confirmed by surgery and histopathology. Comparisons of miRNA expression in different endometriotic lesions was restricted to cases with single lesion types to exclude potential influences of burden of

disease on expression. Finally, in contrast to previous studies[89,91,108,122–124,127,129,177] we used multiple reference genes to normalize miRNA expression.

Some limitations with the current study include the lack of diversity in study subjects (predominantly Caucasian). Study participants were predominantly stage III-IV disease and thus the relevance of our findings for women with earlier stage disease (Stages I-II) is unknown. Furthermore, the type of lesions collected (red, clear, blue-black, white, or chocolate cysts) was not consistently recorded and thus the effect of lesion activity on miRNA expression cannot be excluded.

4.7 Concluding remarks

In summary, we have shown that miRNA expression differs between the eutopic endometrium of women with endometriosis compared to a symptomatic control population without endometriosis. Moreover, our results demonstrate that miRNA expression is dependent on the type of endometriotic lesion. Taken together, our data suggests that endometriotic lesion types are biochemically distinct. These differences no doubt contribute to the challenges in identifying sensitive and specific diagnostic markers of endometriosis and could provide insight into the mechanisms underlying treatment failures. We suggest that our results support the proposal that endometriosis is a heterogeneous disease.

4.8 Future Directions

Evidence has shown that circulating miRNA is a reflection of the miRNA profile at the tissue level[180,181]. It is therefore logical to move forward and measure these differentially expressed miRNA in the serum of the same study population. Doing this can make for a more reasonable approach to a minimally-invasive diagnostic marker. A potential use of this test is not only to diagnose disease but to identify what type of lesions a woman has and what treatments would be the most beneficial. Additionally measuring specific targets of these miRNAs from literature, or pathway analysis, could provide insight into what the pathways involved in the pathophysiology of endometriosis. The ultimate goal is to be able to provide individualized patient care to each woman, and to minimize the delay between presentation of symptoms and diagnosis of disease.

CHAPTER FIVE: REFERENCES

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