MICRORNA CONCENTRATIONS IN SERUM THROUGHOUT THE MENSTRUAL CYCLE IN ENDOMETRIOSIS

ASSESSING VARIABILITY IN MICRORNA CONCENTRATIONS IN SERUM THROUGHOUT THE MENSTRUAL CYCLE IN WOMEN WITH AND WITHOUT ENDOMETRIOSIS

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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

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

TITLE: Assessing variability in microRNA concentrations throughout the menstrual cycle in women with and without endometriosis.

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NUMBER OF PAGES: 104

LAY ABSTRACT

Endometriosis affects 10% of women within their reproductive lifespan. Since the gold standard for diagnosing endometriosis is laparoscopy, a diagnostic biomarker for endometriosis is of utmost importance. Currently there is no universally acceptable biomarker and circulating levels of miRNA appear to be promising but results have yet to be replicated. Replication of results may be dependent on reference RNA and definitions of control groups.

ABSTRACT

Endometriosis is an estrogen dependent disease characterized by the growth of endometrial epithelium and stromal cells outside the uterine cavity. Lack of a clinical test results in a diagnostic delay of between 6-12 years. Recent studies suggest that miRNAs may be useful diagnostic tools; however, results remain equivocal. Use of different reference miRNA and definitions of control groups are factors postulated to contribute to the inconsistent findings in the literature. Serum samples were collected from women (n=53) undergoing laparoscopic surgery. Reference RNAs and symptomatic vs asymptomatic control groups were studied. Comparisons were made between cases and controls, controls and treated vs non-treated cases, and controls, endometriosis and adenomyosis. Data were compared by Mann Whitney U tests and Kruskal Wallis tests. A p value ≤ 0.05 was considered statistically significant. Our major finding was that reference RNA selection and categorization of patients influenced results. When using the most appropriate reference and comparing to asymptomatic controls, miR-9 was upregulated and miR-451a was downregulated in women with endometriosis. When using the most appropriate reference and comparing to symptomatic controls, miR-9 and 141 were downregulated in women with endometriosis. miR-9 and 141 were also downregulated in women receiving treatment. When using the most appropriate reference and comparing to women with adenomyosis, miR-451a, 20a and 122 were downregulated in women with endometriosis. While miRNA is the newest and has most promise as a diagnostic biomarker, miRNA expression results are extremely sensitive to multiple experimental variables. Literature continues to approach miRNA research using different definitions of control populations and different reference RNA. To replicate results and

advance the search for miRNA as a diagnostic biomarker for endometriosis, a common methodology between labs will be necessary.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. Lauren (Warren) Foster. I am thankful for all of the times Dr. Foster has challenged me to expand my way of thinking and pushes me to always try new things and work harder. I don't believe that I would have the scientific and problem-solving mindset I have today without that mentorship. I am extremely grateful for the support Dr. Foster has given me in my research and in my writing. Dr. Foster supported and encouraged me to become more confident in my work and share my research by submitting abstracts and presenting my findings. This thesis would not have been possible without such a caring and supportive supervisor.

I would also like to thank all the lab-mates I have had over the past couple of years who have helped me and had a huge impact on me throughout my degree. Maria Haikalis, Mike Tsoulis and Chante Ellis kindly helped me get comfortable in the lab. I'm grateful for the help Maria gave me in understanding protocols and getting my project organized. I'm also grateful to all of the undergraduate students I have worked with and had brain-storming sessions with throughout my degree: Megan Vierhout, Christina Ricci, Christie Tan, Jacqueline Lim, Shay Freger, Ryan Chow and Matthew Hamilton. I am grateful to have worked with Anna Leonova and received her support and feedback on all of my thoughts and scientific endeavors. I am grateful to have been able to work with and learn

from both Emerson Brazil and Elahe Papari. I am extremely grateful to Jocelyn Wessels who offered me helpful guidance in my data analysis and rationale.

I would like to thank my committee members, Dr. Derek Lobb and Dr. Chris Verschoor. I am extremely appreciative of the time they have taken to be a part of my master's thesis. I appreciate all of their advice and comments that have helped me to become a better researcher.

Furthermore, I would like to thank Annette Bullen, Dr. Nicholas Leyland and Dr. Sarah Scattolon for assisting with the recruitment of patients and allowing me to interact with patients, attend surgeries and collect samples for the study.

I would also like to extend a huge thank you to all of the study participants who enthusiastically consented to provide samples for the endometriosis study here at McMaster University. Our research would not be possible without their selfless desire to participate.

Finally, I would like to thank my wonderful family and friends whose support has meant the world to me throughout my academic journey. Although I am the first of any generation to obtain a post-secondary education and at times, they did not understand my love of science they were always supportive of my ambitions and I would not be successful today without their unwavering support.

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LIST OF ABBREVIATIONS AND SYMBOLS

α: alpha

β: beta

AUC: area under the curve

BDNF: brain-derived neurotrophic factor

BMI: body mass index

CA-125: cancer antigen 125

CA-19-9: cancer antigen 19-9

Cp: Crossing Point

CRP: C-reactive protein

DIE: deep-infiltrating endometriosis

FGF-2: fibroblast growth factor 2

FLT-1: soluble vascular endothelial growth factor receptor 1

GnRH: gonadotropin-releasing hormone

HDL: high density lipoprotein

HGF: hepatocyte growth factor

IBS: irritable bowel syndrome

IL-4: Interleukin 4

IL-8: interleukin 8

LDL: low density lipoprotein

MRI: magnetic resonance imaging

NSAIDs: non-steroidal anti-inflammatory drugs

OCPs: cyclic oral contraceptive pills

OMA: endometriomas

PACT: protein activator of PKR

PE: Peritoneal lesions

PEDF: pigment epithelium-delivered factor

PID: pelvic inflammatory disease

PON1: serum paraoxonase

rAFS: revised American Fertility Society

RISC: RNA-induced silencing complex

sICAM-1: soluble intercellular adhesion molecule 1

SOD: plasma superoxide dismutase

TNF-α: tumour necrosis factor α

TRBP: TAR RNA binding protein

VEGF: vascular endothelial growth factor

YKL-40: Chitinase-3-like-protein

DECLARATION OF ACADEMIC ACHIEVEMENT

All of the experiments were performed by me.

CHAPTER 1: INTRODUCTION

1.1 What is Endometriosis?

Endometriosis is an estrogen dependent chronic gynecological disease of unknown etiology characterized by the implantation and growth of endometrial glands and stromal cells outside the uterine cavity[1-3]. It is estimated that endometriosis affects 2-10% of reproductive age women (18-40 years) but has even greater prevalence in women with pelvic pain and/or infertility[2]. Indeed, 25-45% of women with painful periods, 20-40% of women with infertility and 35-50% of women with pain and unexplained infertility have been diagnosed with endometriosis[4-6]. Estimations of the prevalence of endometriosis are likely lower than the actual prevalence of endometriosis due to delays in diagnosis and symptom overlap with similar diseases[7-9,360].

The hallmark features of endometriosis are infertility and pain[12-14]. Between 40-50% of women with endometriosis experience infertility and 50% of women with endometriosis report having pelvic pain[12-14]. Painful symptoms of endometriosis include non-menstrual pelvic pain, dysmennorhea (menstrual pain), dyspareunia (painful intercourse), lower abdominal pain and back pain. Altered bowel habits as well as dyschezia (painful bowel movements) and dysuria (painful urination) are also common in women with endometriosis[10,11]. Endometriosis is thought to result in an increase of inflammation and nerve innervation which is thought to be responsible for pelvic pain as well as lowering the quality of oocytes and the receptivity of the endometrium for embryonic implantation[15-17]. In addition to these symptoms,

endometriosis is the third leading cause of hysterectomies in the United States. Along with the painful and disruptive symptoms of endometriosis, endometrial lesions that are left undiagnosed and untreated can also transform and develop into cancerous cells which has been seen specifically in endometriomas that transformed into ovarian cancer[18,19]. Symptoms of endometriosis significantly reduce quality of life and are a major cause of disability in women with endometriosis[20]. However, some women with endometriosis are asymptomatic, and the disease is only diagnosed when they are trying to become pregnant and are unable to conceive[12,21,22].

The cost of treating and managing endometriosis is equivalent or greater than many other common chronic conditions such as migraines, asthma or Crohn's disease[23]. Resources spent on endometriosis are described as direct costs, such as medication and treatment, as well as indirect costs, such as time lost at work or travelling to appointments. In the United states, $22 billion dollars are spent annually on treatment and patient care[8,23,24]. It is estimated that $12,118 per woman are spent on direct costs associated with endometriosis and $15,737 per woman are spent on indirect costs associated with endometriosis[25]. Eighty-seven percent of money spent on endometriosis in the United States is spent on surgery[26]. In Canada, $1.8 billion dollars are spent annually on treatment and patient care[27].

Carl Rokitansky was originally known as the "discoverer of endometriosis" however the first to describe the morphology of the disease was Thomas Cullen and the first to name it was John A Sampson[28-31]. Thomas Cullen described uterine adenomyoma, ovarian endometriosis and deep endometriosis as one disease

characterized by the presence of adeno-myomatous tissue outside of the uterine cavity[30]. We now describe these as three separate lesion types that may be heterogenous in nature as women may have more than one lesion type and each lesion type may behave differently as biochemically distinct entities[347].

Peritoneal lesions (PE) are found on the peritoneum where they are able to implant and proliferate due to the vast supply of sub-peritoneal blood vessels and lymphatics[33]. These lesions rely heavily on these networks for angiogenesis that allows the endometrial glands to invade the surrounding tissue[34,35]. Peritoneal lesions start as microscopic foci that evolve into early-active, advanced and healed lesions[36-40]. PE are dynamic as their appearance changes and the lesions appear and vanish with time [41]. In general, early-active lesions are red and glandular or vesicular[36-38]. They are the healthy and proliferating endometrial glands. Advanced lesions appear black and puckered due to a cycle of partial shedding and regrowth during menstruation that traps intraluminal debris and minimizes vascularization to the lesion[35-38]. Healed lesions appear white and fibrotic from many cycles of cell growth and cell death that eventually cuts off all vascularization to the lesion and leaves only the white plaques and old collagen[35-38].

Endometriomas (OMA), or ovarian endometriosis, are usually made up of a pseudocyst that forms at the site where the ovary adheres to the parametrium[42,43]. This invagination creates a perfect place for regurgitated menstrual debris, a thin layer of endometrium-like tissue, to collect and form a cyst[43]. The invaginated cortex, or wall of the cyst, may be covered partially or fully by these endometrial cells which is why

surgical treatment of OMAs requires complex and personalized surgical treatment plans[42,43].

Rectovaginal, or deep-infiltrating endometriosis (DIE), is the most common, most painful and deepest form of endometriosis[44-46]. It was originally suggested that DIE was the result of the natural evolution and progression of PE but it is now thought that DIE is an entirely separate entity with a unique pathogenesis due to their ability to invade the myometrium without stromal cells, their ability to produce 95% more severe pain, and their lack of recurrence and progression [34,35,45-51]. They are common in younger women and recently have been suggested to arise embryonically lying dormant until puberty on onset of cyclical steroid stimulation from the ovary[355,356]. DIE lesions have a structure similar to adenomyomas in that they can be nodular or polypoid masses, but they involve the posterior vaginal fornix [30,46,52,53]. The depth of a DIE lesion is strongly correlated with pain levels[46,51,54].

1.1.1 Pathogenesis of Endometriosis

Endometriosis has an unknown etiology with two main theories on its pathogenesis: the theory of retrograde menstruation and the theory of metaplasia. It is thought that a variety of several factors including trauma, hormones, heredity and dysfunction of endometrial cells and immune response play a role in the development of endometriosis.

The theory of retrograde menstruation was the first theory on the pathogenesis of endometriosis. It was proposed by John A Sampson who stated that endometrial cells

were regurgitated through the fallopian tubes during menstruation, implanted onto nearby pelvic structures and proliferate, forming ectopic lesions as seen in endometriosis[31]. This theory has been strengthened by the findings that women with endometriosis have shorter cycle lengths, longer durations of menstruation with heavier menstrual flows, more endometrial cells in their abdominal cavity when fallopian tubes are flushed and a retrograde pattern of contraction during their menstrual phase rather than an antegrade pattern[55-57]. There is also a higher prevalence of endometriosis in women with congenital menstrual outflow tract obstruction[58,59]. Similarly, if a cervical stenosis is experimentally induced in non-human primates, endometriosis occurs as a result[60]. However, retrograde menstruation occurs in approximately 90% of women but endometriosis develops in only a small subset of these women[357]. Therefore, factors other than retrograde menstruation must be important in the pathogenesis of this disease. Consequently, several other theories and ideas have been proposed on the pathogenesis of endometriosis.

The theory of metaplasia has three proposed methods of action: the coelomic metaplasia hypothesis, the Mullerian hypothesis, and the induction hypothesis. The coelomic metaplasia hypothesis states that the original celomic membrane is able to undergo metaplasia and form endometrial glands and stroma with stimulation from hormones and immunological factors[61,62]. This explains how pre-pubertal girls can develop endometriosis before they have had retrograde menstruation or exposure to estrogen[62]. However, there are reports of early prenatal menstruation which may underlie the formation of deep-infiltrating endometriosis[355,356]. The Mullerian hypothesis states that residual cells from the Wolffian or Mullerian ducts may persist and respond to

estrogen upon puberty. These cells may then proliferate and behave as active lesions[62,63]. This hypothesis is imperfect since there are endometriotic lesions found outside of the Wolffian and Mullerian tracts[64]. The induction hypothesis states that the hormones and immune factors in the pelvic cavity may cause undifferentiated cells lining the peritoneum to differentiate into endometrial glands and stromal cells[65-67]. This hypothesis has been strengthened by a study that found peritoneal cells could be transformed into Mullerian type cells from hormone exposure[68].

It is believed that multiple specific factors are involved in the pathogenesis of endometriosis. Trauma is believed to influence the pathogenesis of endometriosis since endometrial cells do not adhere appropriately to the epithelium when it is intact[69]. Hormones play a large role in the implantation and growth of ectopic lesions. Ectopic lesions are driven to proliferate by estrogen and proliferation is inhibited by progesterone[68,71]. Women with endometriosis have increased responsiveness to estrogen, a higher bioavailability of estrogen and resistance to progesterone and these factors combined play a major role in the development and proliferation of ectopic implants[62,68,70-73].

Genetic predisposition is thought to be important for the development of endometriosis. Women have a 6x higher risk of developing endometriosis if they have a first degree relative with severe endometriosis[74]. Monozygotic twin studies have also shown that there is a high concordance rate of developing endometriosis as well as concordance between the stage of endometriosis developed[75]. Genetics are thought to influence immune regulation and behaviour of endometrial cells[45,62].

Dysfunctions are observed in the endometrial cells and immune response of women with endometriosis. When endometrial cells are regurgitated into the pelvic cavity, an immune response is triggered that increases inflammation[76]. This elevation in inflammatory cytokines and growth factors encourages implantation and angiogenesis which allows further establishment of endometriotic implants[77]. Endometrial cells in women with endometriosis favour anti-apoptotic pathways and pro-proliferation pathways[78-81]. Cells which are resistant to apoptosis are also more resistant to natural killer cells (NK) and therefore evade the immune system which fails to eliminate them[82-84]. Deficiency in cellular immunity and NK ability is thought to promote the implantation and proliferation of endometriotic implants[82,85-89].

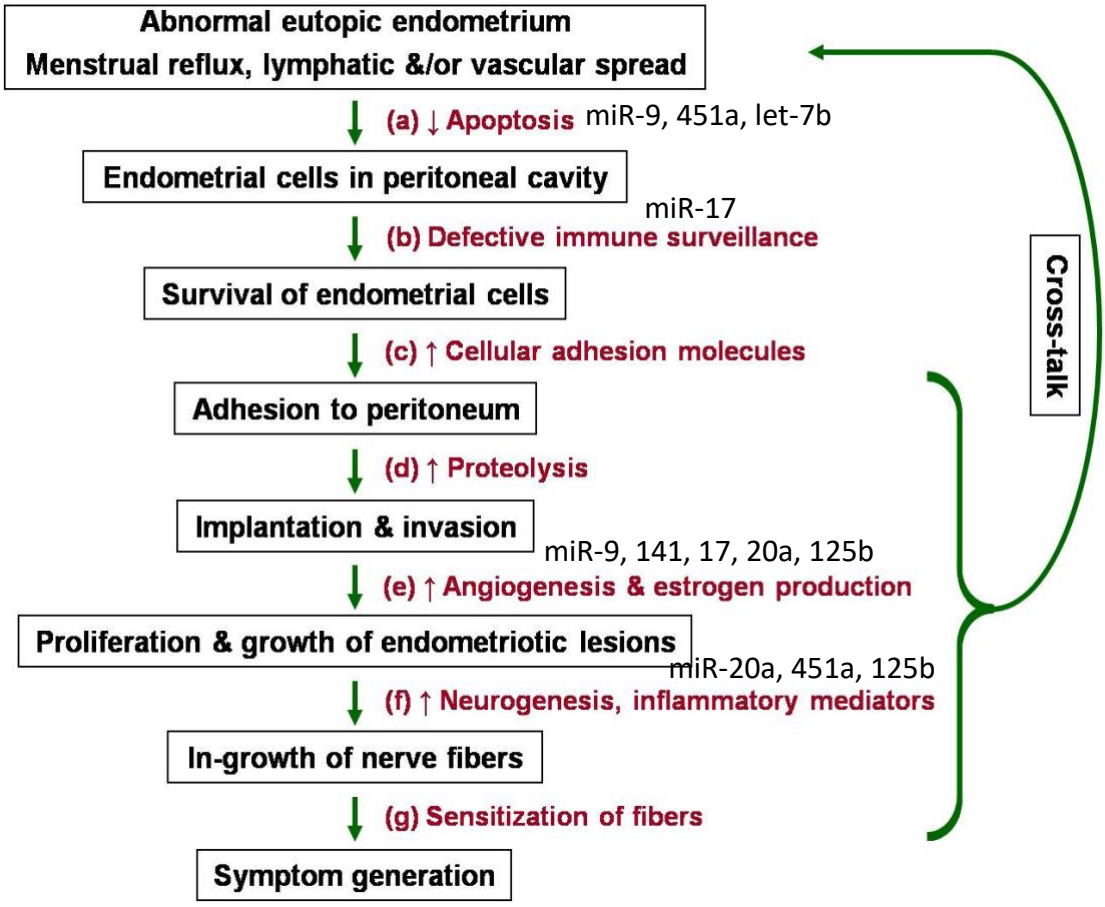

Figure 1. Processes, pathways and miRNA involved in the development of endometriosis. (Modified from Hey-Cunningham, A. J., Peters, K. M., Zevallos, H. B., Berbic, M., Markham, R., & Fraser, I. S. (2013). Angiogenesis, lymphangiogenesis and neurogenesis in endometriosis. *Front Biosci (Elite Ed)*, *5*, 1033-56.)

1.2 Current Treatments

There are two approaches to manage the symptoms and implications of endometriosis: medical management and surgical management. Medical management focuses on reducing excessive inflammation, suppressing ovarian cycles and inhibiting the effect of estrogen. Surgical management is the act of removing an identified endometriotic lesion or removing the affected pelvic organ. Neither medical or surgical

management are capable of providing long term or universally effective relief of symptoms and are therefore suboptimal[17,64,90,91].

There are many medications currently used in the medical management of endometriosis that are chosen based on the patients' needs. The most common forms of treatment include non-steroidal anti-inflammatory drugs (NSAIDs), cyclic oral contraceptive pills (OCPs), progestins, androgens, gonadotropin-releasing hormone (GnRH) agonists, GnRH antagonists and aromatase inhibitors.

NSAIDs such as Advil and Motrin are used for pain management. These medications reduce pain without suppressing ovarian function[92]. OCPs such as Alesse are used to reduce pain by suppressing ovarian function[92]. Progestins can be taken orally (Visanne) or inserted vaginally (LNG-IUD). They reduce pain by inducing a state of pseudo-pregnancy which prevents ovulation, proliferation and the secretion of cytokines[93,94]. Androgens such as Danazol suppress ovarian function by inhibiting gonadotropic substances[95-97].

Gonadotropin-releasing hormone (GnRH) agonists such as Lupron are injections that regulate the menstrual cycle by lowering estrogen levels and preventing lesion growth[17,92,98]. Add-back therapy is required to manage the side effects of low estrogen levels[98,99]. GnRH antagonists such as Elagolix reduce pain and suppress ovarian function by preventing the action of gonadotropins, creating a similar environment to GnRH agonists but more quickly[96,100]. Aromatase inhibitors such as Letrozole reduce pain and suppress ovarian function by preventing the conversion of androgens to estrogen which reduces bioavailability of estrogen and suppresses lesion proliferation[62,96,101].

The current strategy for successfully treating endometriosis involves suppressing the production and bioavailability of estrogen and inducing amenorrhea to create an environment that, with minimal estrogen, inhibits the proliferation of implanted lesions and the progression of disease[102]. The main limitations of these current treatments are that they are suppressive rather than curative and they are contraceptive rather than fertility-enhancing. Medications used to treat endometriosis give temporary relief and symptoms return after treatment is stopped[96].

The surgical management of endometriosis is done through laparoscopy. Laparoscopy is the use of a carbon dioxide laser which can remove and excise peritoneal lesions and endometriomas as well as lyse any visualized adhesions[17,103]. It is a topic of debate whether surgical excision is an effective method of treatment. Surgical excision of lesions may reduce the risk of progression in disease severity as well as progression to cancer but it may also promote disease recurrence and adhesion formation leading to compromised fertility[104,105]. Laparoscopy is currently the gold standard for the diagnosis of endometriosis with a sensitivity of 0.94 and a specificity of 0.79[358]. It is a poor method for diagnosis due to its inherent risks as a surgical procedure, the latency in diagnosis due to doctors' reluctance to suggest surgery, and observer bias of the surgeon when determining a surgical diagnosis[121-125].

1.3 Current Diagnosis & Characterisation Methods

Ultrasounds, along with magnetic resonance imaging (MRI) have been useful in detecting endometriomas and deep infiltrating endometriosis but have also mis-diagnosed

women with endometriosis as being endometriosis-free[107-110]. Ultrasounds are not reliable in identifying the presence of endometrial tissue or the stage of disease[106].

Diagnosing endometriosis requires 3 conditions to be met: there must be endometrial structures outside of the uterine cavity, these structures must be endometrial glands and stromal cells and the cells must be benign, or non-cancerous[111]. Combining laparoscopy with histological confirmation allows these criteria to be assessed and are the gold standard for diagnosing endometriosis[17]. The symptoms of endometriosis overlap with several similar conditions (e.g., irritable bowel syndrome (IBS), pelvic inflammatory disease (PID), adenomyosis and ovarian cysts) and are also variable between women[7,8,112-114]. Due to the variability in clinical presentation of endometriosis, direct visualization is necessary to confirm diagnosis and many women with similar symptoms will undergo this surgery[115,116].

When visualizing the lesions during surgery, surgeons have several different classification systems to determine and describe the extent of the disease. The most common classification system is the revised American Fertility Society (rAFS)[117]. Stages of endometriosis are classified according to multiple criteria that combine histopathological and anatomical information to distinguish the three lesions types (peritoneal, ovarian and deep-infiltrating)[117,118]. This method of classification focuses on the extent of the disease, the number of lesions and the severity of these lesions and the rating, or staging, given is a reflection of how difficult the surgery has been and how likely conception will be[51,117]. The other two classification systems are the Acosta, which places high emphasis on OMAs and the Enzian score, which is used to classify deep-

infiltrating endometriosis but needs further refinement for appropriate and universal classifications to be made[119,120].

1.3.1 Challenges with current diagnosis and classification systems

There are challenges presented with the diagnosis and classification of endometriosis at a few levels. These challenges arise with the laparoscopy itself, but also with the classification system and even with the reliability of histological confirmation.

Although laparoscopy is a minimally invasive surgery, it still has its potential hazards and risks. Organ damage, hemorrhage, blood vessel injury, bowel injury, infection, adhesion formation, anesthetic complications, travel, financial costs and time away from work and family are all drawbacks of the procedure[121,122]. These risks of complications result in a large delay between the onset of symptoms and a definitive diagnosis since doctors are reluctant to suggest surgery[123,124]. Pain is often managed with medication before surgery is suggested which delays diagnosis and allows the disease to progress to a more severe state[125,126].

The American Fertility Society has made significant modifications to their scoring system in hopes of standardizing their classification system; however, there still exists the limitations created by observer error which limits reproducibility[127,128]. The objective staging determined by the surgeon often poorly reflect clinical symptoms[128]. The rAFS classification system also puts very little weight on deep-infiltrating endometriosis and emphasizes the impact of the disease on fertility rather than pain[51,117].

The reliability of histological confirmation of lesions is limited by the technical efficiency in the retrieval and processing of lesions[115]. Any damage done to biopsies or excess tissue removed with lesions can alter the diagnosis. Only 70-75% of lesions discovered and removed in surgery are confirmed by histology to be positive for endometrial glands and stromal cells[129].

Due to these challenges with surgical complications and subjective scoring and diagnosis of endometriosis, it would be ideal to have a quantitative and non-invasive diagnostic marker.

1.4 Clinical Markers for the Diagnosis of Endometriosis

Currently, there are no effective laboratory tests that can diagnose or confirm a diagnosis of endometriosis[130,131]. Many groups are searching for a reliable semi-invasive or, ideally, non-invasive diagnostic test. A semi-invasive test would require a sample of peritoneal fluid, a transvaginal ultrasound-guided aspiration, or a sample of endometrial tissue obtained after a transcervical endometrial biopsy[110,121]. A minimally invasive diagnostic test requires either serum, plasma or menstrual fluid[110,121]. Blood can be easily obtained from a patient and allows repeatable and high-throughput measurements when searching for a diagnostic marker[132]. Blood has been extensively studied but no single biomarker or panel of biomarkers has been replicated effectively yet in multiple populations to suggest its universal use as a diagnostic biomarker of endometriosis[110,116,121]. A non-invasive method would be analysis of urine[110,121]. Very few

studies have been done on urine but there are varying levels of protein expression in cases and controls[108,133,134].

Discovering a diagnostic biomarker for endometriosis has so far proven elusive[135]. An effective diagnostic marker requires a high level of specificity, the ability to detect the disease in early stages, and should be non-invasive. However, a diagnostic marker is currently not available[110,116,121,136]. While panels of markers are able to offer more sensitivity and specificity, they have yet to be validated[132,137,138]. The World Endometriosis Society recommends that a top research priority in endometriosis is the discovery and development of a non-invasive diagnostic test to reduce health care and individual costs associated with endometriosis[1,110,124]. Approaches to date have been made in several fields and progress has been made on a few specific pathways.

Table I. Previous approaches at identifying a diagnostic biomarker for endometriosis.

Approach	Attempts	Strengths	Weaknesses
Phenomics	Correlation of characteristics with endometriosis diagnosis	Determination of at-risk populations without collecting any samples from patients invasively or non-invasively	Symptom overlap with similar diseases makes a definitive diagnosis impossible
Genomics	Dysregulation of gene expression in women with endometriosis	Pin-pointing of specific genetic sequences involved in development of endometriosis gives information on affected pathways in endometriosis	Failure to replicate results, complex protocols and expensive equipment
Proteomics	Differential protein expression in women with endometriosis	Pin-point specific proteins involved in development of endometriosis gives information on affected pathways in endometriosis	Failure to replicate results, expensive and time-consuming
Metabolomics	Differential metabolite concentrations in women with endometriosis	Least invasive test quantifying urine samples, gives information on affected pathways in endometriosis	Requires large volumes of urine, expensive equipment and is time-consuming

1.4.1 Phenomics

Phenomics is the study of characteristics that happen to correlate with a diagnosis of endometriosis. Identifying these phenomena allow us to understand which features a woman has that may put her at a greater risk for developing endometriosis. Medical history, symptom report, pelvic exam and transvaginal ultrasound to find links between characteristics and diagnosis of endometriosis contribute to the phenomic profile[139,140].

Primary subfertility, irregular menstrual cycles, dysmenorrhea, chronic pelvic pain, obesity, use of OCPs and being a non-smoker are predictors of endometriosis[141]. Women with low body weight, alcohol use, freckles, benign skin growths, higher sensitivity to sun exposure, an early menarche, short cycle length and heavy menstrual cycles were more likely to have endometriosis[140,142]. Women with high body mass index (BMI) were more likely to have deep-infiltrating endometriosis[143]. Women with endometriosis also have a higher risk of having other immune disorders including fibromyalgia, chronic fatigue syndrome, allergies, infections, ovarian and breast cancer, melanoma and non-Hodgkin lymphoma[144-149].

There have been several attempts at creating a diagnostic model based on the patients' presentation of symptoms and their history of menstrual cycles, alcohol and cigarette consumption, however, predicting a diagnosis based off of symptoms was found to be unreliable due to the overlap of symptoms for endometriosis with a variety of similar diseases[150,151].

Although this approach may allow the determination of at-risk populations and does not require the collection of samples invasively or non-invasively, it is unable to

quantitatively diagnose endometriosis especially when it needs to be differentiated from diseases with similar clinical presentation.

1.4.2 Genomics

There is a significant difference in gene expression within the endometrial transcriptome of women with endometriosis. Many genes are dysregulated in endometriosis and these changes can be described using comparative genomic hybridization microarrays which allow us to delineate endometriosis from other benign gynecological conditions and have been suggested to be able to determine disease stage[152-158].

It has been hypothesized that the genetic alterations found in women with endometriosis are responsible for the poor immune clearance and the altered behaviour of endometrial cells that result in the development of endometriosis[45,62].

The ability to pin-point a specific gene sequence responsible for the development of endometriosis would be extremely helpful for understanding the pathogenesis of endometriosis and developing more effective treatment options with fewer adverse side effects. Since results require expensive equipment and complex protocols, results are not reproducible and would produce many challenges as a universal biomarker for diagnosis.

1.4.3 Proteomics

Unique protein fingerprints and proteomic profiles have been found in the tissue of women with endometriosis [116,159-161]. A proteomic fingerprint model based on three

peptide peaks had a sensitivity of 91.4% and a specificity of 95% for diagnosing endometriosis. This same proteomic fingerprint was validated in another cohort of women with endometriosis[160]. A proteomic fingerprint model with five peaks which included fibrinogen β-chain peptide was also explored and had a sensitivity of 88% and a specificity of 84% to diagnose endometriosis during the menstrual phase [161].

Similar to genomics, proteomics could pin-point specific proteins involved in the pathogenesis of endometriosis that could give information on the pathogenesis of endometriosis and how to treat it more effectively. Unfortunately, proteomics also requires expensive and time-consuming technology that is not yet standardized to allow for universally reproducible results[110,116,121].

1.4.4 Metabolomics

Several metabolite concentrations and metabolite ratios have been studied in women with endometriosis in hopes of discovering a metabolite that could be used as a diagnostic biomarker for endometriosis. Stearic acid, glucose, isoleucine and arginine have been found to be significantly lower in women with endometriosis[162,163]. Lactate, 3-hydroxybutyrate, alanine, glycerophosphatidyle choline, valine, leucine, threonine, 2-hydroxybutyrate, lysine and succinic acid were found to be significantly higher in women with endometriosis[163]. In one study, 81 metabolite ratios and eight metabolites were significantly higher in women with endometriomas[164].

While metabolomics requires the least invasive form of sample collection, the robust volumes of urine required and expensive and time-consuming equipment needed to

determine the metabolites produced and pathways affected in the patient make this a poor option for diagnosis.

1.4.5 Physiological Markers of Endometriosis

1.4.5.1 Protein Biomarkers

Several proteins have been studied as diagnostic biomarkers of endometriosis. Some studies suggest that they are good markers of disease while other studies find them poor indicators of disease[116,137,165-173,345,346]. Glycoproteins, cancer antigen 125 (CA-125), cancer antigen 19-9 (CA-19-9), follistatin, zn-α2-glycoprotein and glycodelin A have been explored as diagnostic biomarkers of endometriosis[116,137,165-173,345,346].

CA-125 has been studied alone as well as in panels with several other markers; however, results have low levels of sensitivity and specificity for diagnosing endometriosis as well as poor accuracy overall to distinguish endometriosis patients from controls and women with other gynecological conditions. Although correlations have been found between CA-125 expression and disease severity in endometriomas, CA-125 is not a good marker because it is not specific to endometriosis [116,137,165-171].

CA-19-9 has been studied on its own as well as coupled with CA-125; however, it is a poor marker for endometriosis for similar reasons to CA-125[168]. Not only does it have a lower sensitivity and specificity for diagnosing endometriosis but it is also a marker of ovarian tumours[116]. Follistatin is a protein that inhibits activin. It has been found to be elevated in women with endometriosis, especially in women with endometriomas[116,172]. However, these results have not been reproduced by others[173]. Zn-α2-glycoprotein and

glycodelin A have also been identified in higher concentrations in women with endometriosis but lack reproducibility and the required sensitivity and specificity of an effective diagnostic marker[345,346].

1.4.5.2 Inflammatory Markers

Many cytokines have been studied as diagnostic markers of endometriosis; however, it is unclear whether or not they are able to discriminate between women with endometriosis from women with other gynecological conditions with inflammation[174].

Interleukin 4 (IL-4), Chitinase-3-like-protein (YKL-40), C-reactive protein (CRP) and co-peptin were found to be significantly upregulated in women with endometriosis although these results have not been replicated[116,175-178]. A panel of markers including interleukin 8 (IL-8), tumour necrosis factor α (TNF-α) and CA-125 was able to differentiate 201 women with endometriosis from 93 women without endometriosis with a sensitivity of 0.1 and specificity of 0.84 in women with moderate-severe endometriosis and a sensitivity of 0.87 and specificity of 0.71 in women with minimal-mild endometriosis which closely matches the diagnostic potential of laparoscopy[179]. Although these results are promising for clinical diagnosis of endometriosis, the diagnostic markers are weak since they rely on expensive equipment and time-consuming procedures and detection of inflammatory markers will be different in both chronic and acute inflammatory diseases.

1.4.5.3 Angiogenic Markers

Angiogenesis is extremely important in the development of endometriosis. Endometriotic implants rely on angiogenesis to supply the lesion with the hormones and growth factors required to invade surrounding tissue, implant and proliferate[180,181]. Angiogenic markers and proteins have been studied as diagnostic biomarkers of endometriosis.

Angiogenic factors were found to have increased expression in the tissue of women with endometriosis[182-187]. Vascular endothelial growth factor (VEGF) was upregulated in women with endometriosis and plays a role in the progression of disease[184,188-192]. Following Danazol treatment, women had elevated levels of VEGF in their plasma[193] but following surgical excision of lesions, VEGF-A was significantly lower suggesting that VEGF-A expression may play an important role in angiogenesis for lesion survival[194,195]. Fibroblast growth factor 2 (FGF-2), angiogenin and soluble vascular endothelial growth factor receptor 1 (FLT-l) were elevated in the serum of women with endometriosis[116]. Supporting the notion that angiogenesis plays an important role in endometriosis, pigment epithelium-delivered factor (PEDF), an inhibitor of angiogenesis, was found to be significantly lower in women with endometriosis[196]. Hepatocyte growth factor (HGF) was also lower in women with endometriosis but results were not replicated[116,197,198].

1.4.5.4 Other Markers

Markers of oxidative stress have been studied as diagnostic biomarkers of endometriosis. Due to the retrograde flow of menstruation and the increased volume of fluid in the pelvic cavity of women with endometriosis, oxidative stress may be higher from erythrocytes that release iron when they rupture[199]. Serum paraoxonase (PON1), high density lipoprotein (HDL) and plasma superoxide dismutase (SOD) were found to be significantly lower in women with endometriosis[200,201]. Total cholesterol, triglycerides, low density lipoproteins (LDL), lipid peroxidases, 25-hydroxycholesterol and vitamin E were significantly higher in women with endometriosis[202-204].

Cell adhesion and invasion proteins have been studied as potential diagnostic markers of endometriosis. Soluble intercellular adhesion molecule 1 (sICAM-1) was studied in a panel with 3 other markers to diagnose endometriosis and has been found to rise during Stage I-II of endometriosis and decline during Stage III and IV[116,205]. Osteoponin, a cell adhesion molecule, as well as matrix metalloproteinases responsible for allowing the invasion of endometriotic lesions were found to be elevated in women with endometriosis[206-210].

Since nerve fibres contribute to pain and dysmennorhea in endometriosis, detection of nerve fibers has also been used to diagnose endometriosis[219-223]. Nerve fibers have been detected in peritoneal lesions and are 14x more dense in women with endometriosis but this may not be useful because contrary results have been reported and the procedure is invasive and technically demanding[221,223].

Neurotrophins are proteins that are involved in the formation and regulation of the nervous system. They are required to help neurons grow and function[359]. There is an important role of neurotrophins in uterine physiology and they are proposed to be important in the pathogenesis of endometriosis[211-216]. Circulating brain-derived neurotrophic factor (BDNF) concentrations are higher in women with endometriosis and levels decline after lesions are removed during surgery suggesting its role in lesion formation and maintenance[217,218].

1.5 miRNA

miRNAs are highly conserved non-coding single-stranded segments of RNA generally 19-25 nucleotides long[224-228]. They are involved in regulating many biological processes through modulation of mRNA translation[278-282]. Although miRNA levels are much lower than mRNA, their ability to bind to several different mRNA transcripts and the ability of several miRNAs to bind to the same mRNA transcripts results in a diverse and complex gene expression network[225,269,319]. miRNA are thought to be good potential biomarkers as they are highly stable in body fluids, resist RNase degradation, are generally very tissue-specific, and have no known post-transcriptional modifications[229,230]. They have been found to be dysregulated within tissue and bodily fluids in several other diseases and strong correlations have been found between tissue and circulating expression which identifies them as a possible non-invasive diagnostic tool[231-235]. miRNAs have been identified and are used as biomarkers for other diseases since they are readily available and stably expressed in circulation and bodily fluids

[235,298-302]. Several circulating miRNAs have been proposed as markers for endometriosis [271,301,303-308].

1.5.1 Biogenesis

The biogenesis of miRNAs is summarized in Figure 2. This process involves the transcription of long primary transcripts which are put through multiple check-points that produce hairpin intermediates (pre-miRNA) that are eventually cleaved into mature and functioning miRNAs[236]. This process is regulated by developmental and/or tissue-specific signaling[237].

Most miRNA transcripts are found within introns and multiple miRNAs may be clustered together in a way that allows transcription of many miRNAs at once as a single polycistronic transcription unit[238-241]. miRNA transcription is accomplished by RNA polymerase II[242,243]. Each cell type may have a different combination of miRNAs expressed and the expression of these miRNAs will often correlate with their hosts' genes[244,245].

Once transcribed, the primary miRNA exists as a hairpin structure within the nucleus[236]. Two proteins, called Drosha and Pasha, form a large complex called the microprocessor complex which cleaves the stem-loop portion of the hairpin structure and releases pre-miRNA, a double stranded RNA duplex[236,246-248]. There is an alternative pathway for the biogenesis of miRNAs that is independent of processing by Drosha. In this pathway, primary miRNA transcripts are cleaved by the lariat debranching system to produce pre-miRNA[249,250].

Processing of pre-miRNA to mature miRNA is completed in the cytoplasm[236]. The pre-miRNA is exported from the nucleus into the cytoplasm through nuclear pores in the nuclear membrane, a process which is mediated by exportin-5[251-254]. Once the pre-miRNA reaches the cytoplasm, it is processed by Dicer to generate two single-stranded miRNAs[255-259]. Each arm of the pre-miRNA duplex, the 3' arm and the 5' arm, becomes a distinct mature miRNA[260].

Based on the thermodynamic stability of each strand of the mature miRNA, one is chosen to be loaded on to Argonaute[261-263]. Argonaute associates with Dicer, TAR RNA binding protein (TRBP) and protein activator of PKR (PACT) to form an RNA-induced silencing complex (RISC) which is ready to bind to and act upon mRNA transcripts[264-266]. The inactive strand of miRNA that was not chosen is held in P-bodies, non-membrane-bound aggregates of several enzymes and mRNA transcripts, to be stored or degraded[267].

1.5.2 Function

The function of miRNAs is summarized in Figure 2. miRNAs are involved in the post-transcriptional regulation of genes. Within the miRNA-RISC complex, miRNA base-pair with the 3' region of an mRNA transcript and induce gene silencing through translation repression, transcript degradation or heterochromatin formation[227,267,268-271]. If the RISC pairs perfectly with the mRNA transcript, degradation occurs while imperfect pairing results in inhibition of translation[270]. These deactivated mRNA transcripts remain bound to the RISC and accumulate in cytosolic P-bodies, similarly to the inactive miRNA

strand, where they can be stored or degraded[267,272]. In some cases, although very rarely, miRNA can bind to mRNA transcripts and encourage their translation[267].

Since miRNAs are released into circulation by cells or shed into circulation when tissue is damaged, there is a strong correlation between miRNA levels in tissue and in circulation[235,273]. Within the circulation, miRNAs are held within membrane vesicles or bound to protein complexes which allow them to bind to and act upon mRNA transcripts in distant cells[274-277].

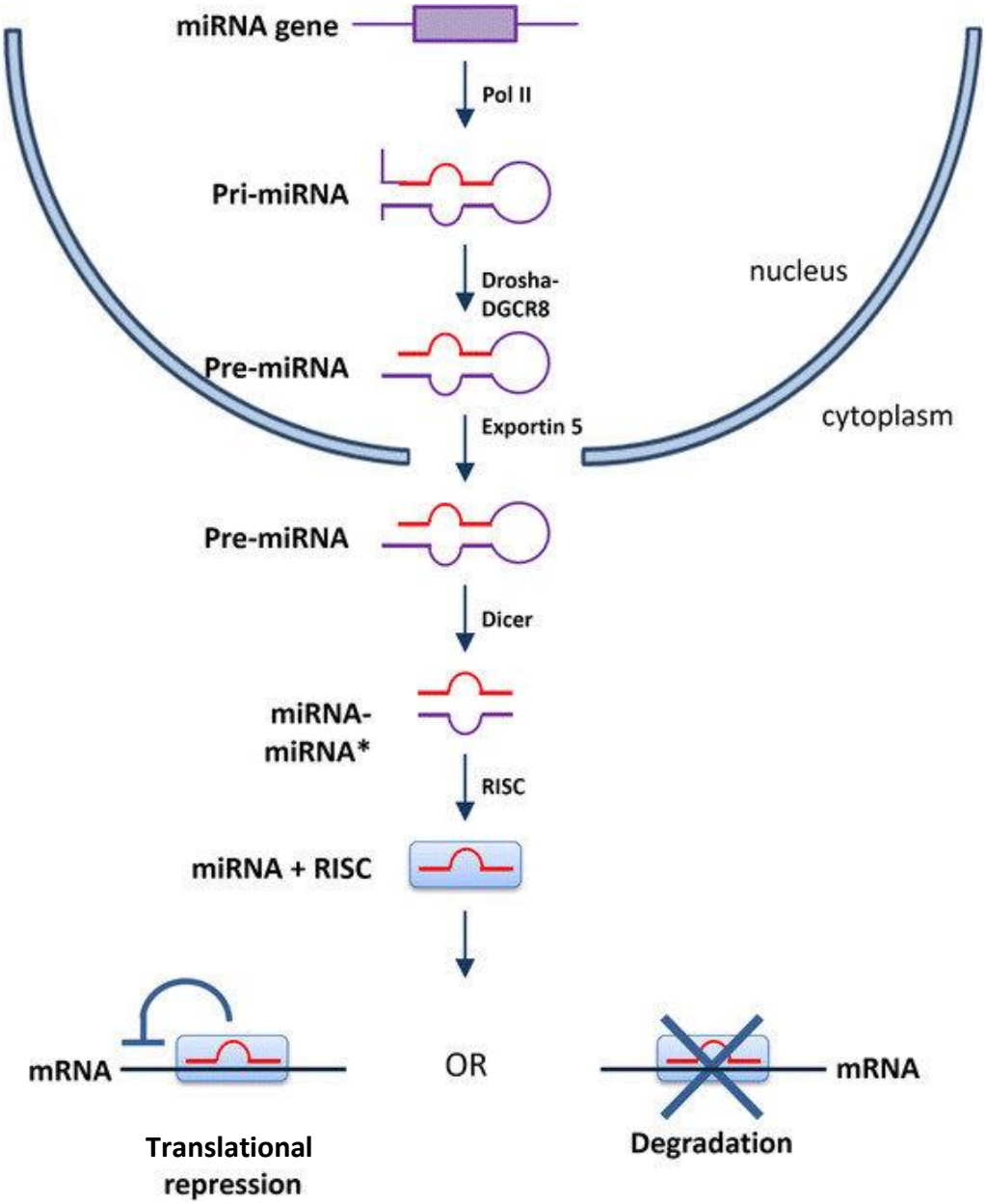

Figure 2. The biogenesis and function of miRNA in humans. (Modified from Devaux, Yvan & Stammet, Pascal & Friberg, Hans & Hassager, Christian & Kuiper, Michael & Wise, Matt & Nielsen, Niklas. (2015). MicroRNAs: New biomarkers and therapeutic targets after cardiac arrest?. Critical Care. 19.)

1.5.3 Involvement of miRNA in endometriosis

miRNAs have been found to be involved in the regulation of many broad signaling pathways where they act as negative feedback[278-282]. Some affected pathways include cell differentiation, migration and invasion, cellular matrix remodeling, inflammation, local estrogen biosynthesis, progesterone resistance and angiogenesis, all of which are important in the development and pathology of endometriosis. Moreover, miRNA may work to alter several pathways in endometriosis at one time[278-281,283-297].

1.5.4 miRNA in tissue

miRNA expression in tissue has been extensively studied in endometriosis. Comparisons have been made between the eutopic tissue of women with endometriosis and women without endometriosis, and between paired eutopic and ectopic tissue in the same woman with endometriosis. Expression of multiple miRNAs found within the tissue were cycle phase dependent[287,309-314].

12 miRNAs that regulate fibrinolysis and angiogenesis were differentially expressed in the eutopic tissue of women with endometriosis compared to women without endometriosis[314]. Several studies have identified dysregulated miRNAs in the eutopic endometrium of women with endometriosis; however, many of these findings have inconsistent comparison groups and results have not been replicated [118,287,292,294,296,314-316]. Many studies have compared the expression of miRNAs in eutopic tissue to expression in ectopic tissue. Several miRNAs have been found to be dysregulated depending on lesion type, however, these findings have not been reproduced as endometriosis is commonly

studied as a hetereogenous disease grouping lesion types though each lesion type is biochemically distinct[287,296,297,312,317,318,347].

1.5.5 Circulating miRNA

Circulating miRNAs are an ideal candidate for a non-invasive diagnostic biomarker. They have been studied extensively in the plasma and serum of women with endometriosis but results have not yet been appropriately replicated.

There are four main studies that have looked at miRNA expression in serum. When comparing cases to symptomatic controls, Wang et al (2013) found miR-199a and 122 to be upregulated and miR-145, 141 and 9 to be downregulated[301]. Cho et al (2015) also compared cases to symptomatic controls and found cycle-dependent downregulation of miR-let-7b, during the proliferative phase, and miR-135a, during the secretory phase[273]. Comparisons between cases and symptomatic controls made by Cosar et al (2016) showed that miR-125b and 451a were upregulated and miR-3613 was downregulated[304]. Nothnick et al (2017) were able to replicate these results for miR-451a when comparing cases to symptomatic and healthy controls. They also found this same upregulation in baboons with endometriosis[305].

Another four studies reported miRNA expression in plasma. When comparing cases to symptomatic controls Jia et al (2013) found miR-17, 20a and 22 to be downregulated[303]. Rekker et al (2015) compared cases to symptomatic and healthy controls and found that miR-200a and 141 were downregulated[306]. Bashti et al (2018) compared cases to symptomatic controls and found miR-145 was upregulated and miR-31

was downregulated[307]. Nisenblat et al (2019) compared cases to symptomatic and healthy controls and found miR-139-3p, 155 and 574-3p downregulated[308].

Although many groups have looked at miRNA expression in the serum and plasma of women with endometriosis, there is very little replication seen which may be due to variability in population, study design and methodology and results in each study suggesting different miRNAs as biomarkers of endometriosis.

Table II. Previous studies on circulating miRNAs in women with endometriosis.

Study	Biofluid	Comparison Groups	Cases	Controls	Reference RNA	Menstrual Cycle	Findings
Wang et al 2013	Serum	60 case, 25 control	all stages all lesions	Symptomatic	RNU6	NS	↑ 199a & 122 ↓ 145, 141 & 9
Cho et al 2015	Serum	24 case, 24 control	Stage III & IV with PE and/or OMA	Symptomatic	RNU6	P vs S	↓ let-7b (P) & 135a (S)
Cosar et al 2016	Serum	24 case, 24 control	Stage III & IV with PE and/or OMA	Symptomatic	RNU6	NS	↑125b & 451a ↓ 3613
Nothnick et al 2017	Serum	41 case, 40 control	all stages with PE and/or OMA	Symptomatic (20) & Healthy (20)	RNU6	NS	↑451a
Jia et al 2013	Plasma	23 case, 23 control	Stage III & IV	Symptomatic	miR-16	Not studied	↓ 17, 20a & 22
Rekker et al 2015	Plasma	61 case, 65 control	all stages with PE and/or OMA	Symptomatic (35) & Healthy (30)	miR-30e & 99a	NS	↓ 200a & 141
Bashti et al 2018	Plasma	55 case, 23 control	all stages, lesion type not stated	Symptomatic	miR-103-3p	Not studied	↑ 145 ↓ 31
Nisenblat et al 2019	Plasma	variable	Stage I-II or III-IV	Symptomatic (10, 27 & 39) & Healthy (8)	RNU6 vs miR-16 vs miR-30b	NS	↓ 139-3p, 155 & 574-3p

1.6 Challenges with miRNA

There are several challenges when using miRNA expression as a diagnostic biomarker for endometriosis. miRNA themselves are very complex and regulate several hundred mRNA transcripts in redundant and overlapping regulatory pathways[225,269].

Several miRNA can regulate one target together in a complex regulatory network[269,319]. In addition to regulating many targets and many miRNAs regulating the same target, the expression of miRNAs is extremely dynamic and easily influenced by several factors including age, ethnicity, the presence of other diseases, smoking and environmental factors[320-325].

Several miRNAs that have been studied in women with endometriosis have also been found to be dysregulated in other inflammatory and autoimmune disorders, making it hard to specifically identify endometriosis and differentiate it from diseases with similar clinical presentation[326-330]. Studies on miRNA as a diagnostic marker of endometriosis lack reproducibility which may be due to the variability in population but also study design and methodology. Research groups have variable approaches in studying circulating miRNAs (Table II) including the biological specimen studied (serum vs plasma), reference RNAs employed (RNU6, miRs) as well as the classification of case and control groups (lesion type, disease stage, symptomatic vs healthy controls).

There is very little agreement on the best way to normalize miRNA values and which reference RNAs are best[331,332]. The most commonly used reference is RNU6 which has been shown to be unreliable. RNU6 is unstable and its expression varies greatly depending on how samples were processed and stored[277,331,333]. Consequently, new approaches to normalize miRNA values have been suggested and include miRNA spike-in controls as well as endogenously expressed miRNAs[338,339].

In addition, control groups can be defined many ways. Previous literature has defined control groups as either symptomatic or asymptomatic. Symptomatic controls have been

defined as women with severe pelvic pain and infertility but without endometriosis. Asymptomatic controls have been defined as women who are self-reportedly healthy with no history of pelvic pain[271,301,303-308]. There is also a group of women who may be referred to as asymptomatic cases who have endometriosis without symptoms. Variability is seen in the control groups used and the definitions of these control groups.

Subject characteristics and heterogeneity of endometriosis is also not often considered in most studies on circulating biomarkers of endometriosis. Women with and without symptoms as well as with different lesion types are often grouped together as cases even though there has been evidence showing their biochemical differences[347].

1.7 Hypotheses

Currently, no single marker or panel of markers have been universally accepted for the diagnosis of endometriosis. A non-invasive diagnostic marker for endometriosis continues to be a priority in endometriosis research. Failure to replicate results creates many challenges with identifying a universally acceptable and robust diagnostic biomarker for endometriosis and may be due to variability in study design and methodology most likely related to choice of reference RNAs. Therefore, I hypothesize that miRNA levels will be significantly different in women with endometriosis compared to controls and will vary by reference RNA employed and definition of control groups.

1.8 Objectives

1. Compare miRNA levels using different reference RNA.
2. Quantify miRNA levels in cases and controls.

3. Determine whether miRNA levels dysregulated in endometriosis are affected by hormonal treatment.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Participant Recruitment

96 women attending the women's health clinic at McMaster University Medical Center were approached to participate in this study. Inclusion criteria were women between the age of 18 and 50 undergoing exploratory or benign laparoscopic surgeries. Exclusion criteria were women unable to provide consent, women without endometriosis receiving hormonal treatment and smokers. Laparoscopies were performed between April 2011 to May 2018 either for diagnosis of endometriosis due to pelvic pain or for benign gynecological procedures such as tubal ligation for family planning.

Women participating in the study were asked to fill out questionnaires regarding their gynecological and surgical history as well as any current prescribed or over-the-counter medications used. Questionnaire data was transcribed into an electronic database. Menstrual cycle phases were determined based on the womens' report of the last menses. Stages of endometriosis were determined by the surgeon performing the laparoscopy and confirmed with operative reports, both in accordance with the revised Classification of the American Society of Reproductive Medicine[117]. Pathology reports were used to confirm whether women were positive or negative for endometriosis and other gynecological conditions based on the presence of two of three of the following factors: endometrial glands, endometrial stromal cells and/or hemosiderin. All reports were reviewed by two people independently to verify classification of participants. All study participants provided a written informed

consent and all procedures were carried out in accordance with our IRB approved protocol (12-083T).

2.2 Sample Collection and Preparation

For women undergoing diagnostic laparoscopies due to pelvic pain serum was collected before anesthesia. Women undergoing laparoscopies for other benign gynecological procedures had their serum collected while they were under anesthesia before surgery began.

20mL of peripheral blood was collected from the antecubital vein from women on the day of their surgery into a serum-separator vacutainer. Serum was maintained on ice for 30-45 minutes to allow for appropriate clotting of the blood. Blood was centrifuged for 20 minutes at 1000 G and the serum supernatant was aliquoted by glass pipette into 1-1.8mL volumes and frozen at -80 °C for long-term storage. Serum was stored from 1 month to 7 years.

Serum samples were thawed at 4 °C and the miRNeasy mini kit (Qiagen, Toronto, ON) was used to extract miRNA from the samples. Cel-miRNA-39-3p was spiked into each sample during the extractions to be used as a reference miRNA during qPCR. miScript II RT Kits (Qiagen) were used to synthesize cDNA. Each cDNA synthesis reaction was completed twice for each sample. One cDNA synthesis reaction was required to analyze miRNA levels of the housekeeping gene that was spiked in during extractions, miR-39, and one cDNA synthesis reaction was required to analyze miRNA levels of desired target miRNAs. cDNA synthesis to reverse

transcribe miRNA for miR-39 reference contained 1.5 uL of sample miRNA, 10.5 uL of RNAse-free water, 4 uL of 5x miScript HiSpec Buffer, 2 uL of 10 x miScript Nucleics Mix and 2 uL of miScript Reverse Transcriptase mix. cDNA synthesis to reverse transcribe miRNA for target genes contained 4 uL of sample miRNA, 8 uL of RNAse-free water, 4 uL of 5x miScript HiSpec Buffer, 2 uL of 10x miScript Nucleics Mix and 2 uL of miScript Reverse Transcriptase mix. All reactions were performed in an iCycler Thermocycler (Bio-Rad, Mississauga ON) for 60 min at 37 °C to allow cDNA synthesis followed by 5 min at 95 °C to inactive the reverse transcriptase. cDNA samples were then diluted with 200 uL of RNAse-free water to give a final volume of 220 uL.

2.3 Quantification of microRNA expression

miRNA targets were chosen based on findings from previous literature. All miRNA target primers used can be found in Table III. Reference RNA were selected based on previous literature and suggested normalization procedures. All reference RNA primers used can be found in Table IV. All miRNA expression was quantified using real-time quantitative polymerase chain reaction (PCR) using the miScript SYBR Green PCR kit (Qiagen).

Four different sets of reference RNAs were used as follows: (1) from previous literature: RNU6[273,301,304,305] and miR-16[303], (2) a spike-in control: miR-39 from the miRNeasy extraction kit, (3): suggested normalization procedures: the average of miR-425, 30e and 148b[338,339] and (4) a spike-in control with suggested normalization

procedures: the average of miR-425, 30e, 148b and miR-39. RNU6 and miR-16 are commonly used reference RNAs in circulating miRNA research [273,301,303-305]. miR-39 is a miRNA that was spiked into samples during extractions to control for extraction efficiency. miR-425, 30e and 148b are suggested to be the most stably expressed endogenous normalization miRNAs in serum[338,339].

Table III. Target miRNA primers used.

miRNA target	Company	Catalogue Number	Previous Findings
hsa-miR-9-5p	Sigma (Oakville, ON)	MIRAP00019	downregulated in serum (Wang et al 2013)
hsa-miR-17-5p	Sigma (Oakville, ON)	MIRAP00032	downregulated in plasma (Jia et al 2013)
hsa-miR-200a-5p	Sigma (Oakville, ON)	MIRAP00248	downregulated in plasma (Rekker et al 2012)
hsa-miR-155-3p	Sigma (Oakville, ON)	MIRAP00203	downregulated in tissue (Ricci et al, 2018)
hsa-miR-3613-5p	Sigma (Oakville, ON)	MIRAP00943	downregulated in serum (Cosar et al 2016)
hsa-miR-122-3p	Sigma (Oakville, ON)	MIRAP00133	upregulated in serum (Wang et al 2013)
hsa-miR-141-5p	Sigma (Oakville, ON)	MIRAP00173	downregulated in serum (Wang et al 2013) & plasma (Rekker et al 2012)
hsa-miR-125b-5p	Sigma (Oakville, ON)	MIRAP00138	upregulated in serum (Cosar et al 2016)
hsa-miR-145-5p	Sigma (Oakville, ON)	MIRAP00180	downregulated in serum (Wang et al 2013)
hsa-miR-let-7b-5p	Sigma (Oakville, ON)	MIRAP00004	downregulated in serum (Cho et al 2015)
hsa-miR-451a	Sigma (Oakville, ON)	MIRAP00408	upregulated in serum (Cosar et al 2016 & Nothnick et al 2017)
hsa-miR-22-5p	Sigma (Oakville, ON)	MIRAP00050	downregulated in plasma (Jia et al 2013)
miR-3613-5p	Mobix (Hamilton, ON)	UGUUGUACUUUUUUUUUGUUC	downregulated in serum (Cosar et al 2016)

Table IV. Reference RNA primers used.

Reference RNA	Company	Catalogue Number
cel-miR-39-3p	Qiagen (Toronto, ON)	218819692
Hs-RNU6-2-11	Qiagen (Toronto, ON)	201804030030
hsa-miR-16-5p	Sigma (Oakville, ON)	MIRAP00029
hsa-miR-425-5p	Sigma (Oakville, ON)	MIRAP00391
hsa-miR-30e-5p	Sigma (Oakville, ON)	MIRAP00088
hsa-miR-148b-3p	Sigma (Oakville, ON)	MIRAP00190

Real-time qPCR was completed using the LightCycler480 machine (Roche, Mississauga ON). Each sample was analyzed in triplicates on a 384-well plate. Each well contained a reaction mixture including 1 uL of sample template cDNA, 2 uL RNAse-free water, 1 uL of 10 x miScript Universal Primer, 1 uL of the target or reference RNA primer and 5 uL of 2 x miScript SYBR Green PCR Master-Mix. Amplification was carried out in LightCycler480 with the following cycles: each 384-well plate was heated to 95 °C for 15 min, followed by 45 cycles at 94 °C for 15 seconds, 55 °C for 30 seconds and 70 °C for 30 seconds. Crossing point (Cp) values obtained from these readings were later used for analysis.

2.4 Statistical Analysis

All reference RNAs and combinations of reference RNAs were compared to determine which was the best reference for the current study. Criteria for determining the best reference RNAs included abundance, variability between comparison groups and overall variability. Reference RNA abundance was determined by One-Way ANOVA between all five reference RNAs' Cp values alone or combined (SigmaPlot 11.0). Reference RNA with statistically significantly higher Cp values were considered poor reference RNAs due to low abundance. T tests were performed between Cp values of cases and controls (SigmaPlot 11.0). Reference RNAs with significantly different Cp values between case and control groups were considered poor reference RNAs. Variability was calculated between all Cp values for each

reference RNA (BestKeeper). *p*-values < 0.05 were considered statistically significant.

Target miRNA Expression.

Power of the tests completed were calculated to be 1.0 (SigmaPlot 11.0). The data was assessed for normality and equal variance by the Shapiro-Wilk normality test (SigmaPlot 11.0). Significant outliers were determined by the Grubbs test for outliers (SigmaPlot 11.0).

Comparisons between cases and controls for specific miRNA targets were made using different reference RNAs. All comparisons were made using a Mann-Whitney U test (SigmaPlot 11.0). Comparisons between menstrual cycle stage were made using a Kruskal-Wallis test (SigmaPlot 11.0). Comparisons between asymptomatic and symptomatic controls and between case and control groups and were made using a Mann-Whitney U test. Comparisons between controls, non-treatment and treatment groups and comparisons between controls, endometriosis and adenomyosis groups were made using a Kruskal-Wallis test. *p*-values < 0.05 were considered statistically significant.

Data are expressed as Relative miRNA Expression graphs using Delta-Cp (DCP), $2^{\Delta Cp}$ or $2^{Cp:sample-Cp:reference}$, values for each miRNA. ROCs were generated for all significantly different groups. Sensitivity, specificity, positive predictive values and negative predictive values were calculated for each miRNA target found to be significantly different between groups.

CHAPTER 3: RESULTS

3.1 Population Characteristics

Tissue samples from surgery were sent to the pathology department of the hospital where they were stained and examined to confirm disease (the presence of two: endometrial glands, stromal cells, hemosiderin). After histo-pathological confirmation of disease, sixty-two women were confirmed to have endometriosis, twenty-eight were confirmed to be free of endometriosis and other gynecological conditions and seven women were confirmed to have adenomyosis. The mean age of asymptomatic and symptomatic controls was 36.5 ± 7.6 and 33.6 ± 6.1, respectively. The mean age of cases not receiving hormonal treatment and receiving hormonal treatment was 33.7 ± 6.3 and 34.8 ± 7.0 respectively. The mean age of women with adenomyosis was 41.3 ± 3.1 (Table V). Kruskal-Wallis tests were used to compare demographic variables between groups. Age was not significantly different between groups ($p = 0.180$). Demographic factors such as age of first menses ($p = 0.732$), duration of bleeding ($p = 0.441$) and ethnicity ($p = 0.332$) were not significantly different between groups. Similarly, Menstrual cycle phase ($p = 0.268$) and disease stage ($p = 0.194$) were not significantly different between groups. Most women in the study were Caucasian (59%) and most women with endometriosis had stage III-IV disease (47 of 62).

Table V. Study Population Demographics.

	Asymptomatic Controls (n = 14)	Symptomatic Controls (n = 14)	Endometriosis – no hormonal treatment (n = 25)	Endometriosis – hormonal treatment (n = 37)	Adenomyosis with Endometriosis (n = 7)
Age, mean (±SD)	36.5 ± 7.6	33.6 ± 6.1	33.7 ± 6.3	34.8 ± 7.0	41.3 ± 3.1
Cycle Phase at biopsy, n (%)					
Menstrual	8 (57)	2 (14)	4 (16)	4 (11)	1 (14)
Proliferative	1 (7)	3 (21)	10 (40)	18 (49)	5 (71)
Secretory	5 (36)	9 (64)	11 (44)	15 (40)	1 (14)
Stage, n (%)					
Stage I-II	N/A	N/A	5 (20)	2 (5)	N/A
Stage III-IV	N/A	N/A	18 (72)	29 (78)	N/A
Unknown	N/A	N/A	2 (8)	6 (16)	N/A
Age of first menses, mean (±SD)	12 ± 1.4	13 ± 1.8	12 ± 1.2	12 ± 1.5	12 ± 1.9
Duration of bleeding, mean (±SD)	6.6 ± 2.02	5.7 ± 1.33	5.6 ± 1.66	5.6 ± 1.82	5.9 ± 2.48
Ethnicity, n (%)					
Caucasian	9 (64)	7 (50)	15 (60)	23 (62)	3 (43)
Asian	1 (7)	1 (7)	1 (4)	0	0
Other	3 (21)	1 (7)	2 (8)	4 (11)	2 (28)
Unknown	1 (7)	5 (36)	7 (28)	10 (27)	2 (28)

3.2 Reference RNA Stability, Abundance and Selection

Criteria for determining the best reference RNA included abundance, variability between comparison groups and overall variability. RNU6 ($p < 0.001$) and miR-16 ($p < 0.001$) were both significantly less abundant in all samples than miR-39 and reference miR combinations (Figure 3). Cp values between comparison groups were not significantly different for any reference RNA (results not shown). BestKeeper was used to study variability in reference RNA Cp values. Both the average of miR-425, 148b and 30e as well as miR-425, 148b, 30e and 39 were found to have the lowest variability in Cp values. The abundance and low variability of the combination of miR-425, 148b, 30e and 39 promoted the choice of this combination of miRNAs as the reference for future comparisons in this study.

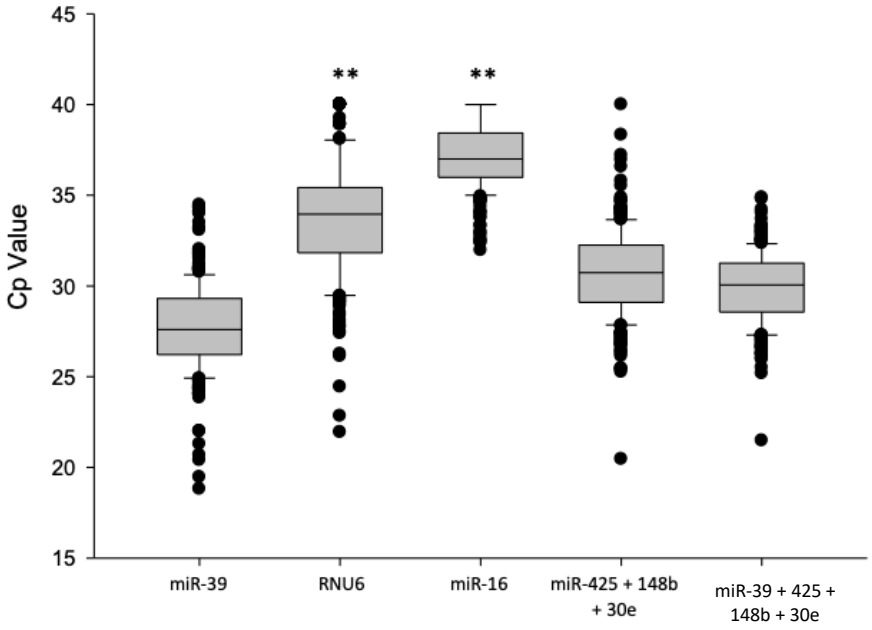

Figure 3. Median Cp values for each reference RNA and combination of miRNAs of interest.

3.3 Menstrual Cycle Phase

Menstrual cycle phase had no impact on miRNA expression levels. miRNA expression for all targets throughout the menstrual cycle (Figure 4). Cycle phases were grouped together for future comparisons made in this study.

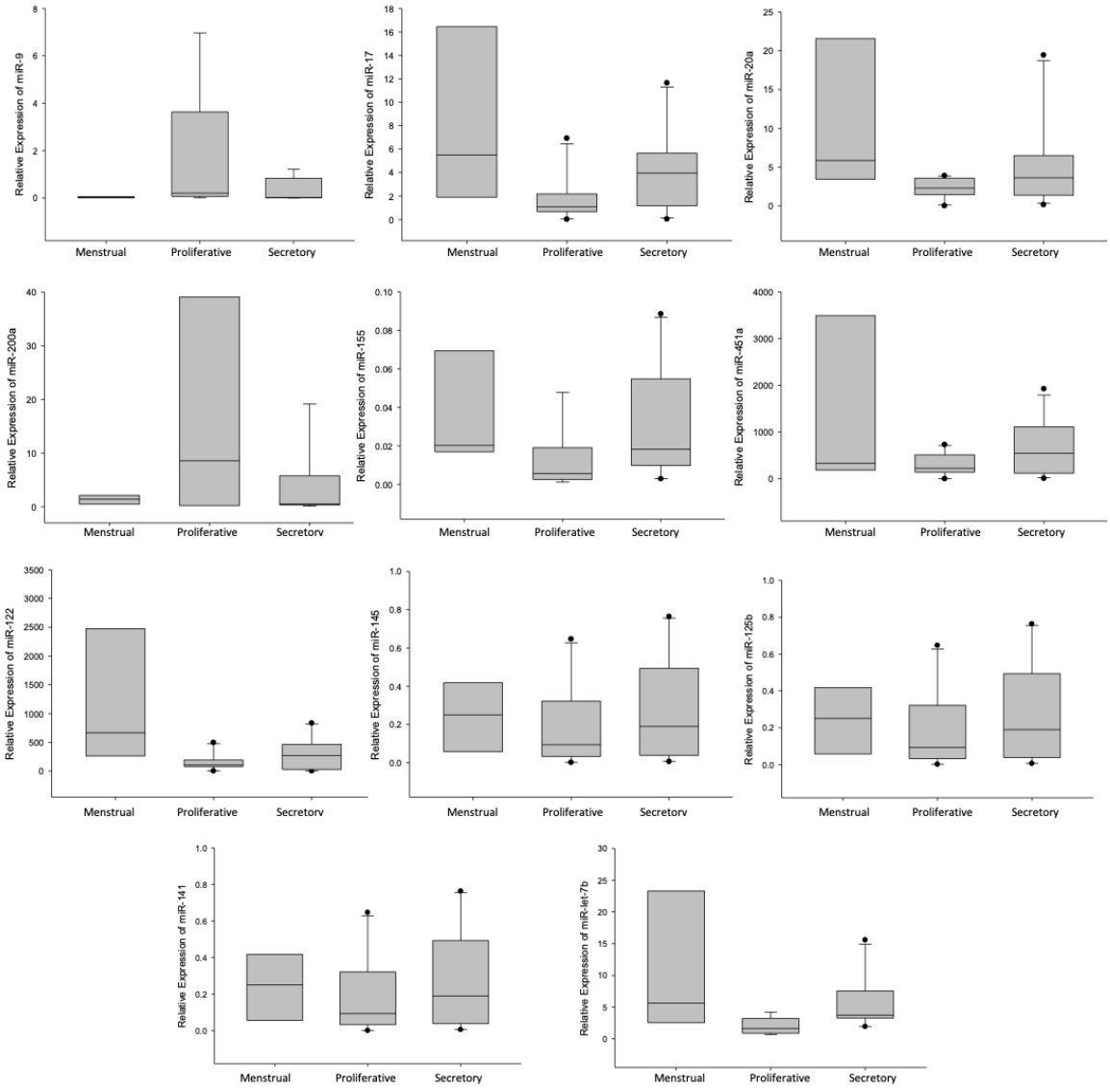

Figure 4. Relative miRNA expression in women with endometriosis throughout the menstrual cycle. Figures show median miRNA expression. Comparisons were made between 4 women in the menstrual phase, 10 women in the proliferative phase and 11 women in the secretory phase. A Kruskal Wallis test was used. P values < 0.05 were considered statistically significant. No significant differences were found between groups.

3.4 Comparing Reference RNAs

The miRNAs chosen for target comparisons are based on the targets and reference RNAs found in previous literature. Several different miRNAs have been used as references in previous studies[271,301,303-308]. Most investigatory studies in serum use RNU6 and miR-16 has been used in plasma[273,301,303-305]. However, these reference RNAs have been found to be unstable, unreliable and poor references for miRNA work in endometriosis[277,331,333-337]. Cel-miR-39 is a spike-in miRNA used to determine extraction efficiency. The average of miR-425, 148b and 30e has been suggested for normalization of miRNAs in serum[338,339]. Comparisons were made between case (not receiving treatment) and control (symptomatic and asymptomatic combined) groups using each reference RNA. Results varied widely in direction and magnitude depending on which reference RNA was employed.

Based on previous literature, specific miRNA target expression with RNU6 as a reference was compared with the average of miR-425, 138b, 30e and 39 as a reference (Figure 5, Figure 6). When using RNU6 as a reference, only miR-125b was upregulated in women with endometriosis ($p = 0.017$). When using the average of 4 miRNAs as a reference, miR-145 expression was unchanged while trend analysis revealed downregulation in women with endometriosis ($p = 0.073$). miR-let-7b expression was also unchanged but trend analysis revealed downregulation in women with endometriosis ($p = 0.078$).

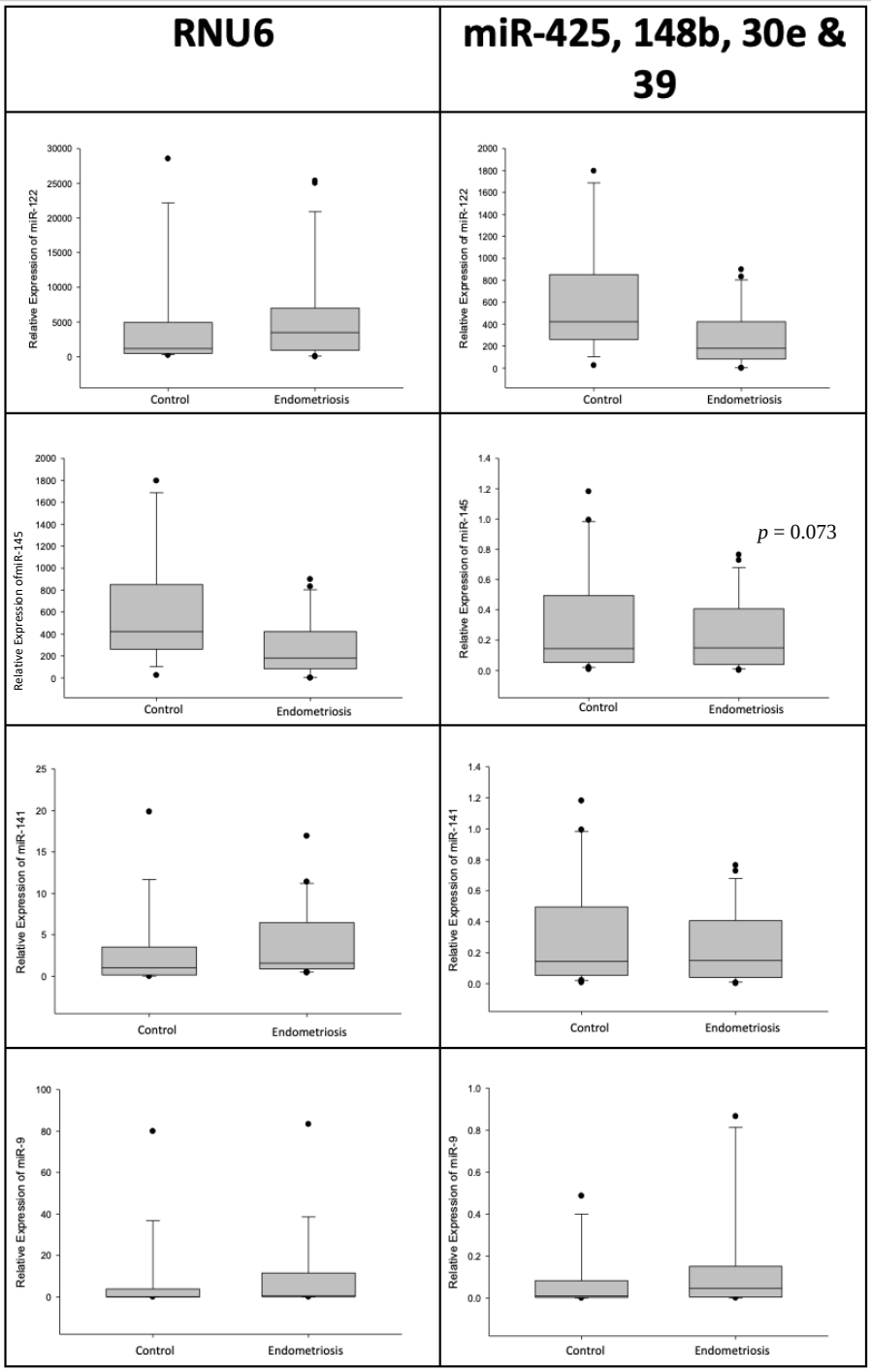

Figure 5. Relative miRNA expression using RNU6 or miR-425, 148b, 30e & 39 as a reference. Figures show median expression. Comparisons were made between 28 women without endometriosis and 25 women with endometriosis not receiving treatment. Mann-Whitney U tests were used. P levels < 0.05 were considered significant. No significant differences were seen between groups. When using the average of miR-425, 148b, 30e and 39 as a reference miR-145 trended towards downregulation in women with endometriosis ($p = 0.073$).

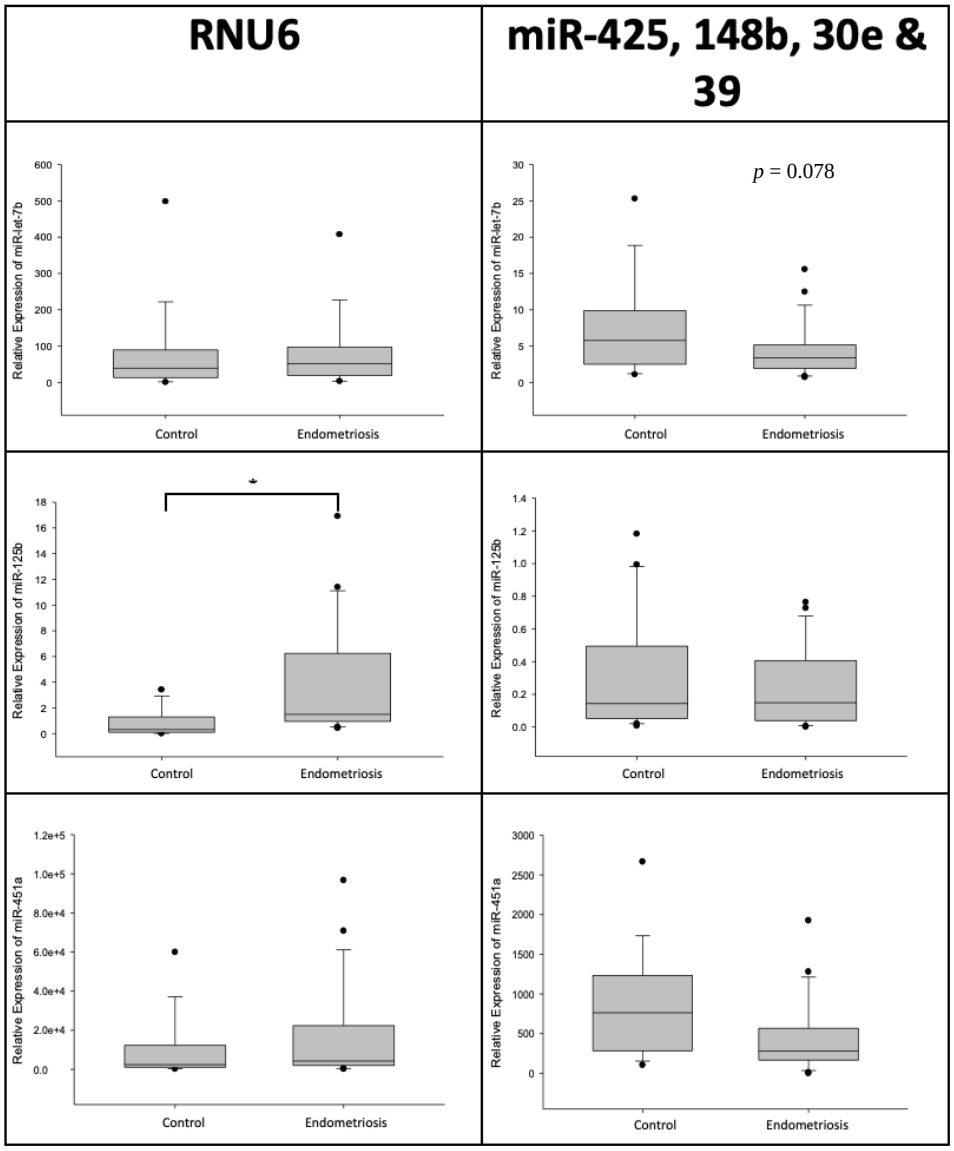

Figure 6. Relative miRNA expression using RNU6 or miR-425, 148b, 30e & 39 as a reference. Figures show median expression. Comparisons were made between 28 women without endometriosis and 25 women with endometriosis not receiving treatment. Mann-Whitney U tests were used. P levels < 0.05 were considered significant. When using RNU6 as a reference miR-125b was upregulated in women with endometriosis ($p = 0.017$). When using the average of miR-425, 148b, 30e and 39 as a reference miR-let-7b trended towards downregulation in women with endometriosis ($p = 0.078$).

Based on previous literature, specific miRNA target expression with miR-16 as a reference was compared with the average of miR-425, 138b, 30e and 39 as a reference (Figure 7). When using both miR-16 and the average of 4 miRNAs as a reference, no significant differences were found between groups.

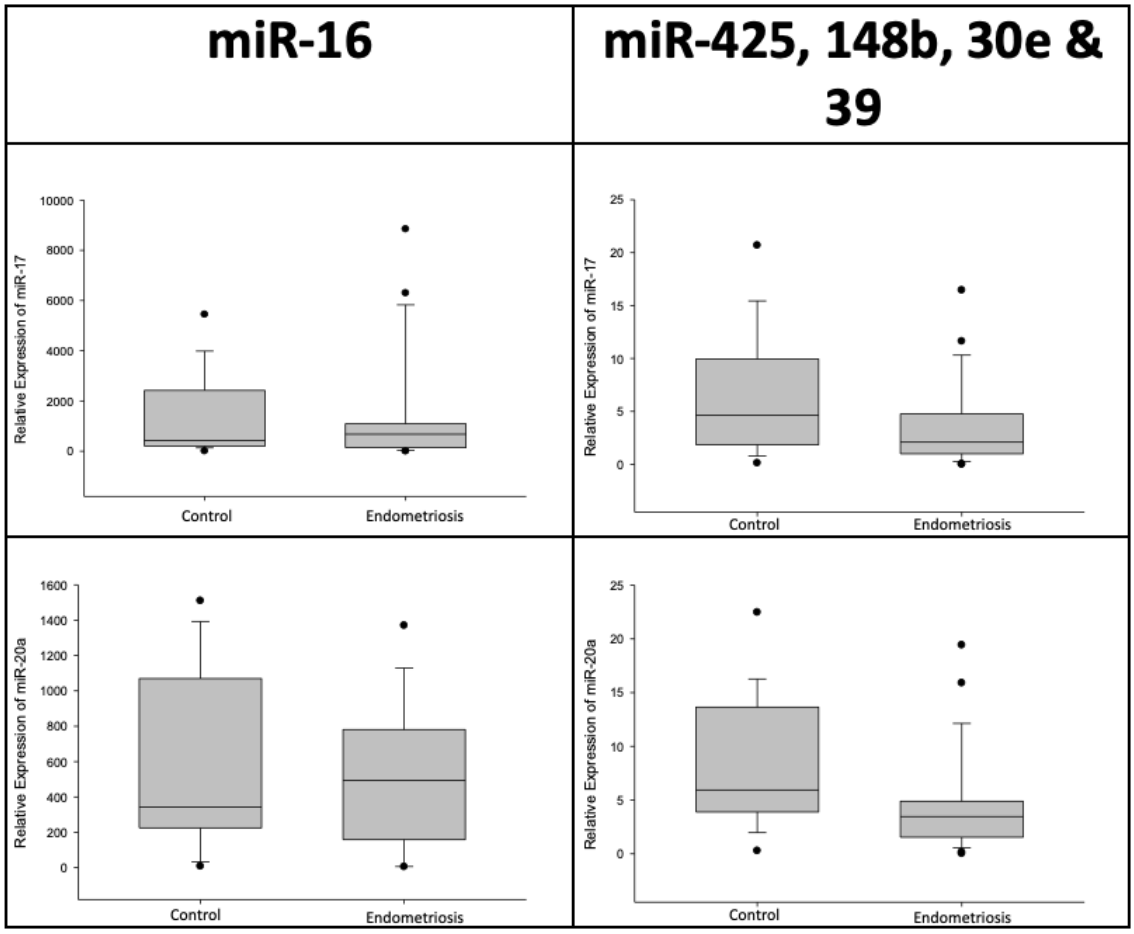

Figure 7. Relative miRNA expression using miR-16 or miR-425, 148b, 30e & 39 as a reference. Figures show median expression. Comparisons were made between 28 women without endometriosis and 25 women with endometriosis not receiving treatment. Mann-Whitney U tests were used. P levels < 0.05 were considered significant. No significant differences were seen between groups.

3.5 Defining Control Groups

Control groups were found to have significantly different Cp values for several miRNA targets (Figure 8) which shows the importance of defining control groups appropriately and making appropriate comparisons. Results varied in direction and magnitude depending on how control groups were defined. Therefore, all subsequent comparisons were made between cases and two separate control groups.

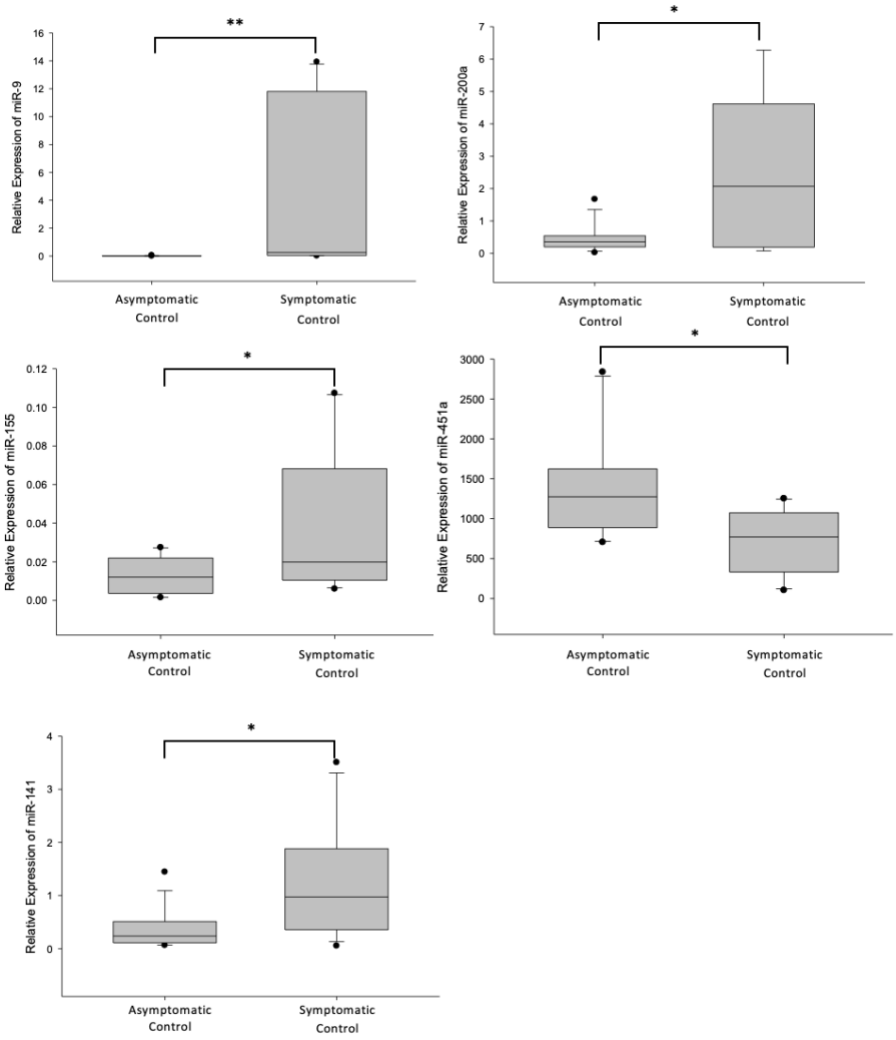

Figure 8. Relative miRNA expression in control populations. Figures show median expression. Comparisons were made between 14 asymptomatic control women and 14 symptomatic control women. A Mann-Whitney U test was used. P values < 0.05 were considered statistically significant. All miRNA targets shown in this figure were found to have significantly different expression between groups.

3.6 miRNA levels in women with Endometriosis compared to controls

3.6.1 miRNA levels in women with Endometriosis compared to asymptomatic controls

Two miRNA targets were dysregulated in women with endometriosis compared to asymptomatic controls (Figure 9). miR-9 was significantly upregulated in women with endometriosis compared to asymptomatic controls ($p = 0.001$). miR-451a was significantly downregulated in women with endometriosis compared to asymptomatic controls ($p = 0.002$). miR-9 had an area under the curve (AUC) value of 0.84 (Figure 10) with a sensitivity of 0.82 and a specificity of 0.69 (Table VI). The positive predictive value (PPV) for miR-9 is 0.8267 and the negative predictive value (NPV) is 0.6822 (Table VI). miR-451a had an AUC value of 0.81 (Figure 10) with a sensitivity of 0.79 and a specificity of 0.79 (Table VI). The PPV for miR-451a is 0.8683 and the NPV is 0.6795 (Table VI).

3.62 miRNA levels in women with Endometriosis compared to symptomatic controls

Two miRNA targets were dysregulated in women with endometriosis compared to symptomatic controls (Figure 9). miR-9 ($p = 0.050$) and miR-141 ($p < 0.001$) were significantly downregulated in women with endometriosis compared to symptomatic controls. miR-451a expression was unchanged while trend analysis revealed downregulation in women with endometriosis compared to symptomatic controls ($p = 0.111$, results not shown). miR-9 had an AUC value of 0.70 with a sensitivity of 0.55 and a specificity of 0.71 (Table VI). The PPV for miR-9 is

0.7721 and the NPV is 0.4671 (Table VI). miR-141 had an AUC value of 0.85 with a sensitivity of 0.72 and a specificity of 0.85 (Table VI). The PPV for miR-141 is 0.8936 and the NPV is 0.6294 (Table VI). miR-9 and 141 have a combined AUC value of 0.89 (Figure 10) with a sensitivity of 0.73 and a specificity of 0.92 (Table VI). The PPV is 0.9490 and the NPV is 0.6562 (Table VI).

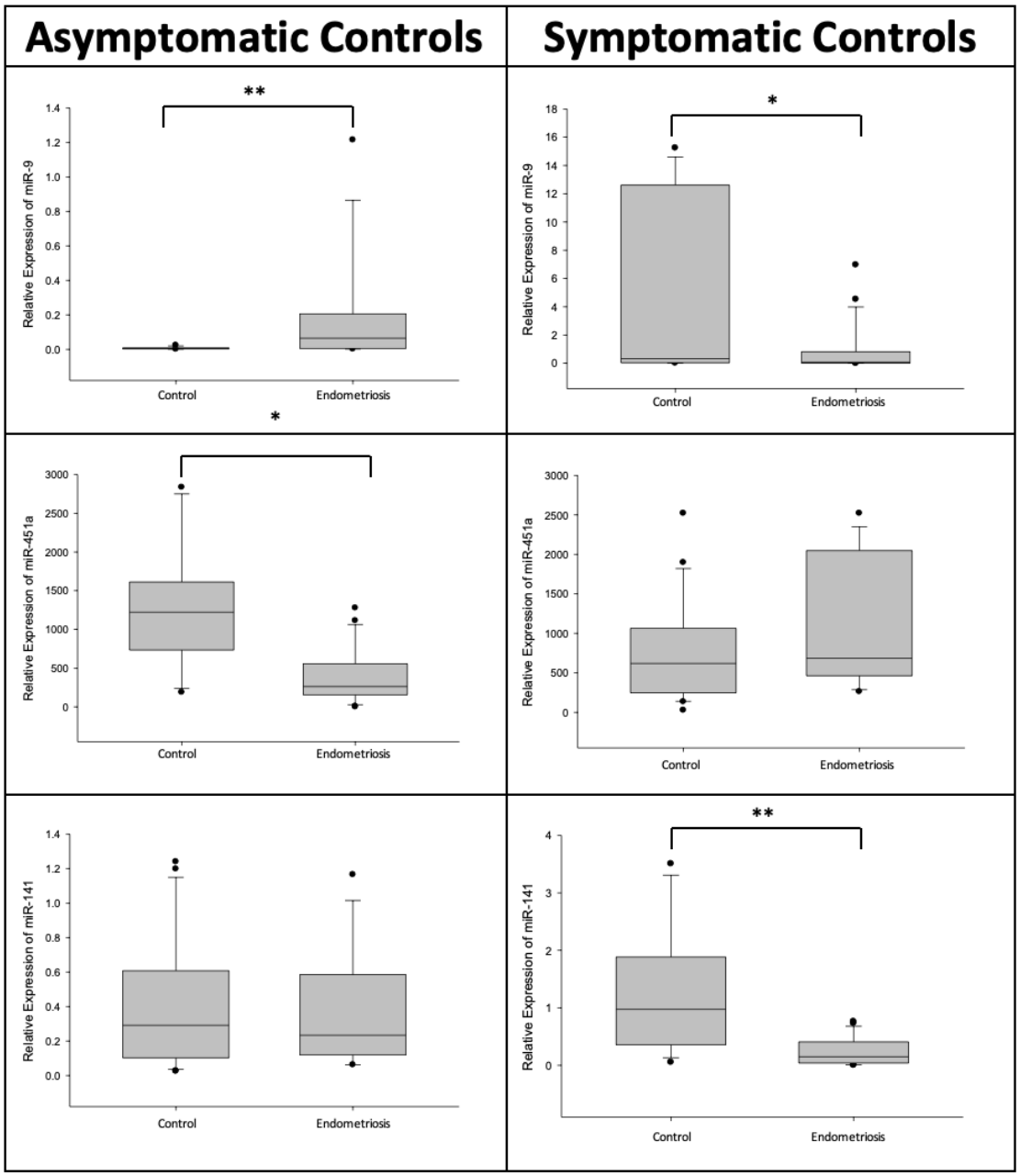

Figure 9. Relative miRNA expression in women with endometriosis compared to women without endometriosis. Figures show median expression. Comparisons were made between 14 asymptomatic or symptomatic controls and 25 women with endometriosis not receiving treatment. A Mann-Whitney U test was used. P values < 0.05 were considered statistically significant. P values < 0.05 *. P values < 0.001 **.

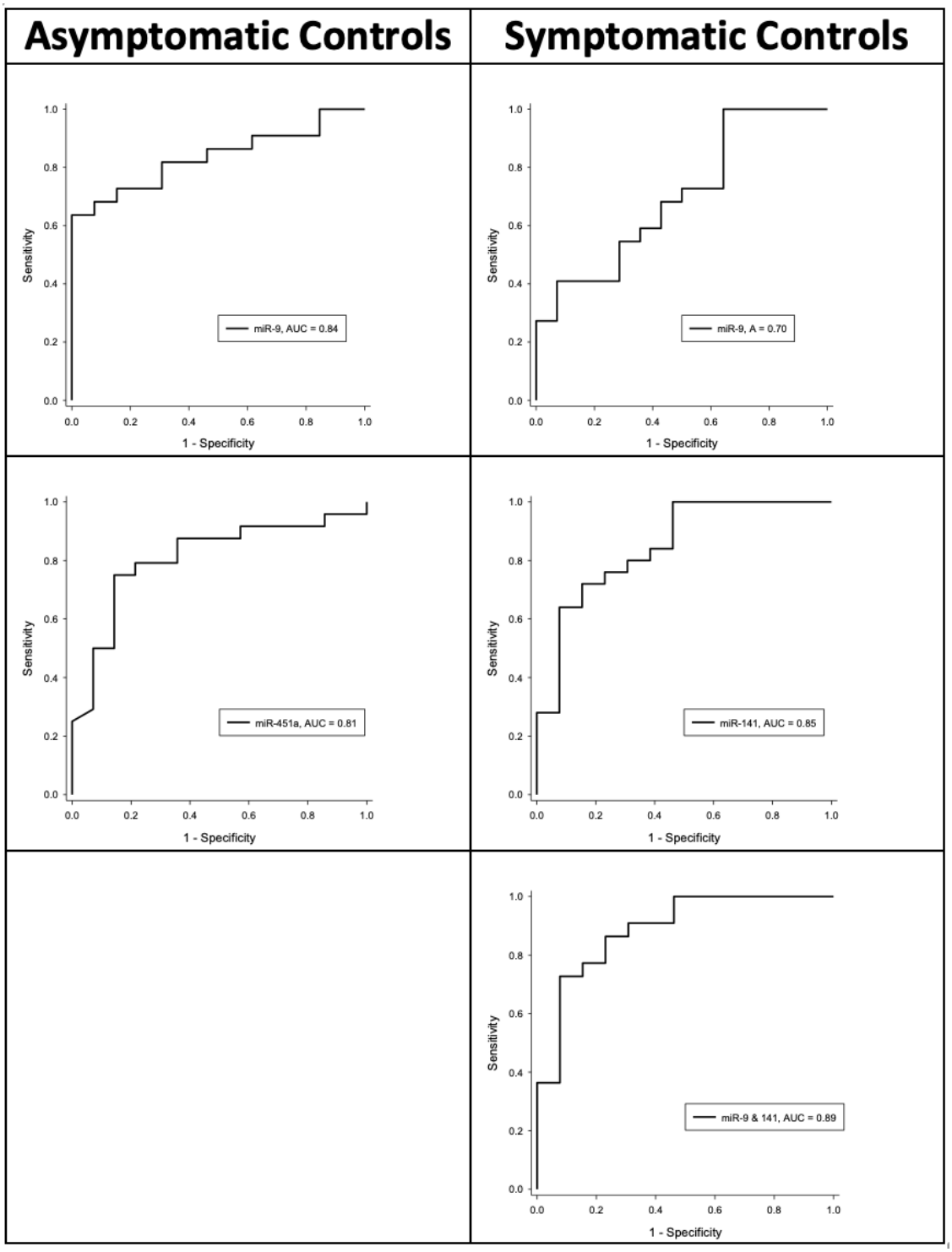

Figure 10. ROCs for target miRNAs that are significantly different in women with endometriosis compared to asymptomatic and symptomatic women without endometriosis.

Table VI. ROC analysis data for significant target miRNAs.

Asymptomatic	miR-9	miR-451a	Symptomatic	miR-9	miR-141	Combined
AUC	0.84	0.81	AUC	0.7	0.85	0.89
Sensitivity	0.82	0.79	Sensitivity	0.55	0.72	0.73
Specificity	0.69	0.79	Specificity	0.71	0.85	0.92
PPV	0.83	0.87	PPV	0.77	0.89	0.95
NPV	0.68	0.68	NPV	0.47	0.63	0.66

3.7 Treatment

It was hypothesized that markers found to be dysregulated in women with endometriosis would be similar to control levels of expression after receiving treatment. This would strengthen the argument that they are consistent markers involved in the pathobiology of endometriosis. miRNA expression was not found to be significantly different between women with endometriosis and women with endometriosis receiving treatment for any miRNA targets studied (results not shown). The expression of miR-9 (compared to both asymptomatic and symptomatic controls) and miR-141 (compared to symptomatic controls) were found to be dysregulated between cases and controls regardless of treatment ($p < 0.05$) (Figure 11). miR-451a was significantly downregulated in cases without treatment compared to asymptomatic controls but not in cases receiving treatment compared to asymptomatic controls ($p < 0.05$) (Figure 9).

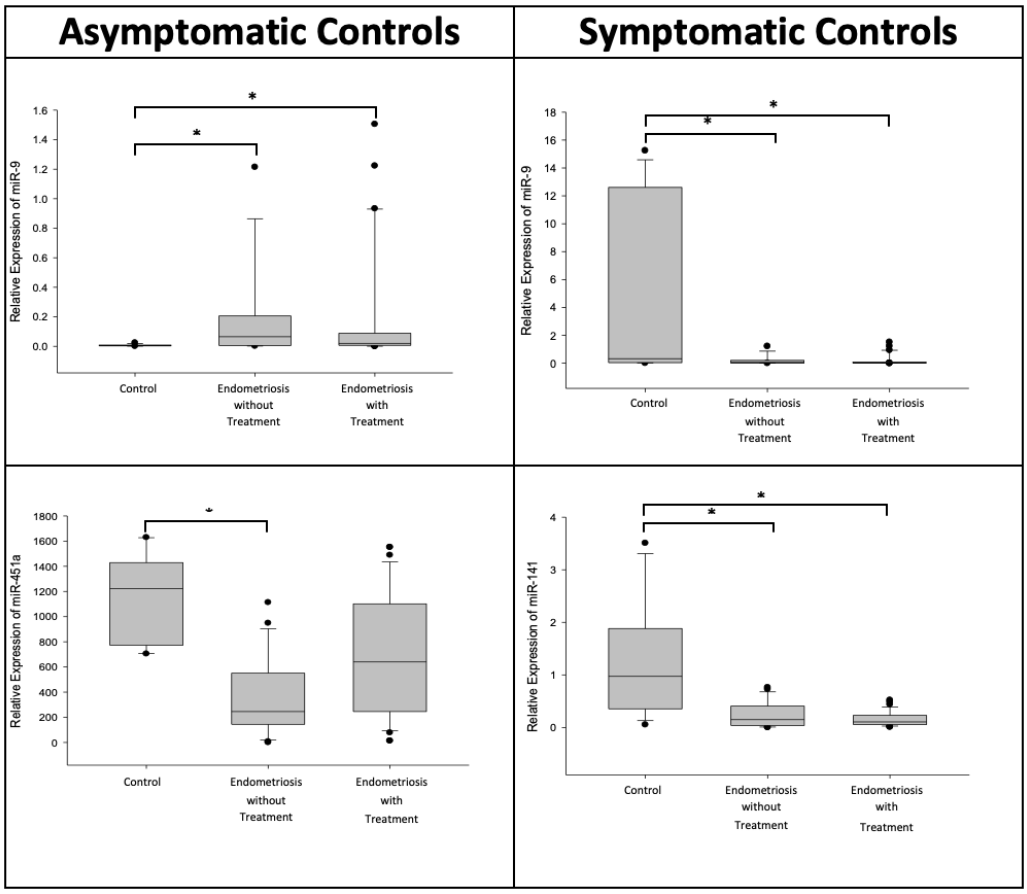

Figure 11. Relative miRNA expression in women without endometriosis compared to women with endometriosis receiving or not receiving treatment. Figures show median expression. Comparisons were made between 14 asymptomatic or symptomatic controls, 25 women with endometriosis not receiving treatment and 37 women with endometriosis receiving treatment. A Kruskal Wallis test was used. P values < 0.05 were considered statistically significant *.

3.8 Adenomyosis

Adenomyosis is a disease commonly within the exclusion criteria of many endometriosis studies. Adenomyosis and endometriosis have similar symptoms and are therefore hard to differentiate before histo-pathological confirmation of disease. In this study, women confirmed to have adenomyosis were not excluded from the study but were compared to symptomatic women with and without endometriosis to determine which miRNAs would be able to differentiate these two diseases with similar clinical presentation. miR-20a (p = 0.005), miR-451a (p = 0.006) and miR-122 (p = 0.009) were significantly downregulated in women with endometriosis compared to women with adenomyosis (Figure 12).

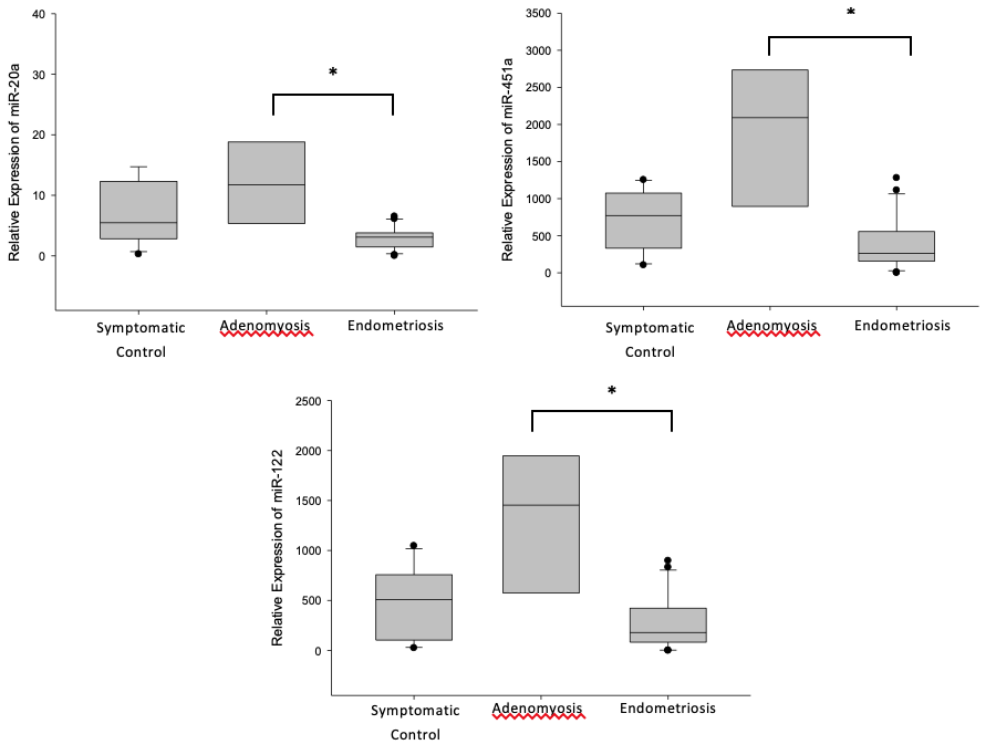

Figure 12. Relative miRNA expression in women with endometriosis compared to women with adenomyosis and symptomatic women without endometriosis. Figures show median expression. Comparisons were made between 14 symptomatic controls, 7 women with adenomyosis and endometriosis and 25 women with endometriosis not receiving treatment. A Kruskal-Wallis test was used. P values < 0.05 were considered significant *.

CHAPTER 4: DISCUSSION

4.1 Summary of Findings

Reference RNA selection in miRNA research is extremely important to produce reliable data that can be reproduced and used as a universally acceptable diagnostic biomarker for endometriosis. My results show the importance of study design in miRNA research when choosing reference RNAs and when defining control groups. miRNA expression was altered in direction and magnitude depending on which reference RNA was employed. The combination of miR-39, 30e, 425 and 148b was the best reference in this study population based on their abundance, variability between comparison groups and overall variability in expression. Women who were surgically and histo-pathologically confirmed to be endometriosis-free had significantly different miRNA expression patterns depending on whether they were symptomatic. Menstrual cycle phase, although relevant when measuring miRNA expression in tissue[287,309-314], appears to have no effect on circulating concentrations of miRNA[301,304-306,308]. miRNAs found to be dysregulated in women with endometriosis in this study were not found to be affected by treatment with the exception of miR-451a, which was downregulated in women with endometriosis without treatment but was not downregulated in women with endometriosis receiving treatment.

The pathogenesis of endometriosis is currently unknown which may be responsible for the broad pathway-based search for diagnostic biomarkers[1-3,130,131]. Without an effective noninvasive diagnostic biomarker, endometriosis continues to be a source of financial and emotional burden with a significant impact on quality of life[8,20,23-

[27]. The latency period between the appearance of symptoms and a definitive diagnosis through laparoscopy is filled with trials on several different medications each with side effects[123-126]. The discovery of an effective biomarker that could differentiate women with endometriosis from women without endometriosis as well as women with other diseases with similar clinical presentation would shorten this latency period, allow for better personalized patient care and reduce morbidity of the disease overall.

The sample size needed to achieve the appropriate power (with 95% confidence) was 34 women. This study contained 96 women and therefore had the power required to find significant differences between groups. miR-9 was dysregulated in women with endometriosis; expression was upregulated when compared to asymptomatic controls and downregulated when compared to symptomatic controls. miR-451a was downregulated in women with endometriosis when compared to asymptomatic controls. miR-141 was downregulated in women with endometriosis when compared to symptomatic controls.

The current gold standard for diagnosing endometriosis is laparoscopy which has a sensitivity and specificity of 0.94 and 0.79, respectively[358]. The best diagnostic marker for differentiating women with endometriosis from asymptomatic women in this study was miR-9 alone with a sensitivity and specificity of 0.82 and 0.69, respectively which does not match laparoscopy. The best diagnostic marker for differentiating women with endometriosis from symptomatic women in this study, which would be the most clinically relevant test, was the combination of miR-9 and miR-141 with a combined sensitivity and specificity of 0.73 and 0.92, respectively which although is closer to that of laparoscopy, is not better.

Although it was hypothesized that a true biomarker of endometriosis would have expression similar to controls after treatment, this was not seen in any of the proposed biomarkers in this study except for miR-451a. This suggests that both miR-9 and miR-141 may not directly be involved in the pathogenesis of endometriosis. Expression of miR-451a was significantly downregulated in women with endometriosis not receiving treatment when compared to asymptomatic controls, however, there was no difference between women receiving treatment and asymptomatic controls.

miR-20a, 451a and 122 were significantly upregulated in women with endometriosis when compared to women with adenomyosis. This suggests that these miRNAs may be useful to differentiate these two groups of women with extremely similar clinical presentation.

4.2 Study Design and Methodology

In this study, study design was explored to determine whether these factors influence miRNA expression. There exists large variability in study design between research groups which may account for why circulating miRNA expression results fail to be replicated[271,301,303-308]. Variability exists in the reference RNAs employed as well as the control groups used (Table II).

RNU6 and miR-16 are reference RNAs that have been previously used in circulating miRNA research. RNU6 is unstable and its expression varies greatly depending on how samples were processed and stored[277,331,333]. miR-16 has been shown to be inconsistently expressed and dysregulated in patients with inflammation, stress and

hepatocellular carcinoma[334-337]. Based on these obstacles, new reference miRNA sequences have been proposed. miR-39 is a miRNA spike-in that comes from C.Elegans and can be used to determine extraction efficiency. miR-425, 30e and 148b are suggested to be some of the best normalization miRNA sequences in serum[338,339]. It has also been suggested that each study should determine its own best reference RNAs through microarray to determine the most stably expressed endogenous control possible[308].

Control groups can be defined many ways. Previous literature[271,301,303-308] has defined control groups as either symptomatic or healthy. Symptomatic controls have been defined as women with severe pelvic pain and infertility but no endometriosis[271,301,303-308]. Healthy controls have been defined as women who are self-reportedly healthy with no history of pelvic pain[271,301,303-308]. Variability is seen between studies in the control groups used and the definitions of these control groups[271,301,303-308]. In this study, controls were defined as symptomatic or asymptomatic. Since asymptomatic controls and symptomatic controls were found to have significantly different miRNA expression, it is important to differentiate these control groups appropriately.

4.3 miRNA Expression

In this study, three clinically relevant comparisons were made: (1) miRNA expression was compared between women with endometriosis and both asymptomatic and symptomatic women without endometriosis, (2) between women with endometriosis receiving hormonal treatment, women with endometriosis not receiving treatment and

women without endometriosis, and (3) between women with adenomyosis, women with endometriosis and symptomatic women with neither.

The clinical relevance of differentiating women with endometriosis from asymptomatic women is to find a test that may be able to diagnose asymptomatic endometriosis when infertility is an issue. The clinical relevance of differentiating women with endometriosis from symptomatic women is to find a test that may be able to differentiate women with endometriosis from women with similar symptoms. Comparisons were made between women without endometriosis, women with endometriosis not receiving treatment and women with endometriosis receiving treatment to confirm that the biomarker is involved in the pathogenesis of endometriosis. Any biomarker that is a true biomarker of endometriosis should return to normal levels of expression following treatment. Comparing women with endometriosis to women with adenomyosis and symptomatic controls gives clinical information on which biomarkers would be able to differentiate between these two diseases with similar clinical presentation.

In this study, when RNU6 was employed as a reference RNA no significant differences between groups were detected apart from the upregulation of miR-125b which is consistent with a previous study[304]. When the average of miR-425, 148b, 30e and 39 were employed as a reference miRNA combination, miR-145 expression was unchanged, but trend analysis revealed it to be downregulated in women with endometriosis. My findings are harmonious with a previous study[301] in which miR-145, 141 and 9 were downregulated in women with endometriosis when compared to symptomatic controls.

Another study[306] also found miR-141 to be downregulated in the plasma of women with endometriosis when compared to both symptomatic and healthy controls. Although both of these markers were found to be differentially expressed in women with and without endometriosis, they were not affected by treatment of endometriosis which suggests that they do not play a crucial role in disease pathogenesis. miR-9 has been found to be upregulated in response to pro-inflammatory cytokines and is thought to be involved in the pathways of angiogenesis, Ras, integrin signaling and actin and tubulin as a tumour suppressor[348-350]. Downregulation of miR-9 was also found in the secretory endometrial tissue of women with endometriosis[294]. miR-141 has been studied in endometrial cancers and was found to be involved in the regulation of hormone receptors; downregulation of miR-141 and 200a increased expression of estrogen and progesterone receptors[354]. miR-122 was upregulated in women with endometriosis in a previous study when compared to symptomatic controls[301] but in this study miR-122 was significantly downregulated in women with endometriosis when compared to women with adenomyosis.

In the present study, miR-let-7b expression was unchanged but trend analysis revealed it to be downregulated in women with endometriosis. There were no significant differences between groups once controls were defined as asymptomatic or symptomatic. These findings contradict a previous study[273] in which miR-let-7b was downregulated in women with endometriosis when compared to symptomatic controls.

In the current study, downregulation of miR-451a was seen in women with endometriosis when compared to asymptomatic women which is in contrast with previous findings[304,305]. Women with endometriosis who were receiving hormonal treatment had

higher levels of miR-451a expression which were closer to the miR-451a expression seen in symptomatic controls. This finding may suggest that this miRNA might play a role in the pathogenesis of endometriosis. miR-451a downregulation was also seen in women with endometriosis when compared to women with adenomyosis. miR-451a has been found to act as a tumour suppressor and is also a marker of hemolysis[351-353]. Expression of miR-3613 was unable to be detected with both bench-top validated primers and a primer generated using the sequence given in the paper that found it to be downregulated in women with endometriosis[304].

In my study I found no significant differences in the expression of miR-17 or miR-20a between groups whether miR-16 was employed as a reference miRNA or whether the combination of miR-425, 148b, 30e and 39 was employed as a reference which is in contrast with previous findings[303].

4.4 Strengths and Limitations

The greatest strength of this study is the use of multiple individual and combined reference RNAs and determination of their abundance, reliability and variability to produce the most accurate possible results in this study group. This idea has not been addressed in other studies until recently in a study which also showed the importance of reference RNA selection and how it impacts miRNA expression[308]. Women in this study were surgically and histo-pathologically confirmed to have endometriosis or to be endometriosis-free by two different individuals which minimizes possible error. The assessment of control groups in this study is novel because women in both control groups

had a laparoscopy and were confirmed endometriosis-free, while other studies looking at two control groups contained asymptomatic controls who were self-reportedly healthy and did not have a laparoscopy. No women with co-morbidities like adenomyosis and no women receiving hormonal treatment were excluded which makes the results more clinically relevant as these are the two main groups of women who require this diagnostic test for endometriosis.

Some limitations in this study include the lack of an endogenously expressed control from within the study population which has been suggested as a strong reference RNA for miRNA research[308]. Another limitation of the study is the lack of diversity as most women were Caucasian and had stage III-IV endometriosis. A third limitation is the possibility of self-reporter error since data on whether women were symptomatic or asymptomatic were self-reported and their menstrual cycle phase was determined from self-reports of their last menses.

4.5 Conclusions

To conclude, this study has shown the importance of study design in the ability to replicate results. Reference RNAs and definitions of control groups impact miRNA expression magnitude and direction and, therefore, are an extremely important factor in creating reliable and reproducible findings. As a noninvasive biomarker is a predominant research need in endometriosis[1,110,124] and replicability has yet to be achieved[273,301-307], it is important to take these factors into consideration.

4.6 Future Directions

A focal point for future research on circulating miRNA as biomarkers for endometriosis should be to normalize study design and methodology across groups. In order for a diagnostic biomarker to be clinically relevant it must be reproducible. While there are standard operating procedures that describe how biological fluids should be collected, processed and stored there is little overlap with how data is collected and analyzed[110,340-344]. Findings in this study show that variability in reference RNAs employed and definitions of control groups have a significant effect on miRNA expression results. Until these factors are controlled for and normalized between groups, reproducibility may not be achieved.

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