**CLINICAL BIOMARKERS FOR THE NONINVASIVE DIAGNOSIS OF ENDOMETRIOSIS**

**CLINICAL BIOMARKERS FOR THE NONINVASIVE DIAGNOSIS OF ENDOMETRIOSIS**

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**ABSTRACT**

Endometriosis is a chronic estrogen-dependent gynecological disease where endometrial cells implant at inappropriate sites causing significant pelvic pain, decreased quality of life, and often infertility. It affects 10% of women of reproductive age, and there is no minimally invasive diagnostic test. Consequently the time to diagnosis, which occurs during laparoscopic surgery followed by pathological confirmation of disease, is prolonged and exceeds 11 years. During this time, the disease often worsens and women thus experience avoidable morbidity. Additionally, endometriosis is a financial burden on the healthcare system, with annual costs of $69.4 billion (U.S.) and $1.8 billion (Canada) in 2009. For these reasons, identifying a clinical marker remains a top priority. Although over 100 putative markers have been identified and reviewed, none have proven sufficiently accurate or reliable for disease diagnosis. For endometriosis to develop endometrial tissue must evade the immune system and adhere, implant, create new vasculature, and grow at ectopic locations. As such it is likely that abnormalities in many or all of these pathways are requisite for disease formation and progression. With this in mind, serum concentrations of eight putative biomarkers believed to be involved in varying pathogenic processes were compared between patients with both surgically and histologically confirmed presence (n=96) and absence (n=25) of endometriosis. Results showed there to be a significant elevation in two of these markers (glycodelin *p*<0.001, and zinc alpha 2-glycoprotein (ZAG) *p=*0.009 when hormonally untreated cases (n=57) were compared to controls. ROC analysis revealed glycodelin to have a sensitivity of 81.6% and specificity of 69.6% for disease diagnosis, while ZAG had a sensitivity of 46% and a specificity of 100%. Subsequent analysis revealed that if combined in panel, using both glycodelin and ZAG could result in a test with a sensitivity of 90%, and a specificity of 65%, giving greater accuracy for disease detection than either independently.

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**DECLARATION OF ACADEMIC ACHIEVEMENT**

All work was conducted by me except for the following: IL-6 assay (Allegra Drumm), RANTES assay (Allana Simon).

CHAPTER 1: INTRODUCTION

**1.1 General Background on Endometriosis**

Endometriosis is a chronic estrogen dependent condition of unknown etiology characterized by the presence of endometrial tissue at ectopic locations including the ovaries, fallopian tubes, gastrointestinal tract, pelvic peritoneum, rectovaginal septum, bladder, and less commonly, the pericardium and pleura1,2. While the exact prevalence remains unknown, largely due to difficulties in diagnosis, it is estimated that 70 million women worldwide suffer from endometriosis, affecting 6%-10% of women of reproductive age3,4. Further reports indicate that up to 50% of infertile women have endometriosis along with 30% of women with chronic pelvic pain5. Endometriosis is most commonly classified according to the revised American Society for Reproductive Medicine (rASRM) classification system, in which the disease is categorized as stage I (minimal), II (mild), III (moderate), and IV (severe) according to disease location and extent of growth. While this is currently the standard classification system, it has received criticism as it relates poorly to levels of pain and infertility in patients6. While newer systems of classification such as the Enzian system and the Endometriosis Fertility Index (EFI) have be proposed, the rASRM classification remains most common in practice6.

**1.2 Diagnosis and Treatment of Endometriosis**

One of the greatest problems with endometriosis is the difficulty of diagnosis. Endometriosis is suspected based on patient history as well as signs and symptoms, is corroborated by physical examination and imaging techniques, and is finally diagnosed only by histological examination of surgical specimens6. While the European Society of Human Reproduction and Embryology (ESHRE) has set out recommendations for sets of symptoms under which physicians should consider a diagnosis of endometriosis, such as dysmenorrhoea, non-cyclical pelvic pain, deep dyspareunia, and infertility, these alone cannot accurately diagnosis the disease as many conditions share similar symptomatology6. Similarly, while imaging techniques such as ultrasound and magnetic resonance imaging can prove useful in heightening suspicions of the disease, they are far from reliable for diagnosis6.

The validity of a diagnostic test is the accuracy at which it can differentiate between people with and without the disease, and is measured by sensitivity and specificity7. Sensitivity measures the proportion of people with the disease that are correctly identified as cases and is also called the true positive rate7. Specificity measures the proportion of people without the disease that are correctly identified as controls and is also called the true negative rate7. Ideally a test should be able to accurately and reliably differentiate between cases and controls with 100% sensitivity and specificity eliminating all possibility of false positives or false negatives. However, this is rarely possible and whatever test has the highest accuracy for a certain disease is termed the gold standard7. All new diagnostic tests must be compared to the gold standard when attempting to be introduced into practice7.

Currently the gold standard and only reliable method of diagnosis is imaging of disease lesions through laparoscopic incision followed by histopathological confirmation of disease5. While effective, this method is far from ideal due to the invasiveness required and the possibilities of complications with surgery. Difficulty in diagnosis has led to a considerable burden of disease, with the mean time between onset of symptoms and disease diagnosis being 11.7 years in the US, and ranging from 4-10 years in various European countries8–10. Due to the invasiveness required for diagnosis, many women for whom there is high suspicion of endometriosis receive analgesics and hormonal medication without a prior definitive diagnosis6. This is particularly common in treating young girls with pelvic pain and dysmenorrhoea6. It is common practice for these patients to receive laparoscopy only if improvements from hormonal contraceptives are not observed in order to exclude or diagnose endometriosis6. However, a response to hormonal treatment does not always indicate presence or absence of endometriosis11,12. Therefore women with other potentially serious estrogen dependent conditions may be misclassified as having endometriosis, and not receive necessary and effective treatments. Furthermore, the use of hormonal contraceptives in young girls increases the risk of a diagnosis of endometriosis later in life13. Thus presumptive treatment of symptomatic women can lead to misdiagnosis in women without the disease, and contribute to the aforementioned delay in diagnosis in women with the disease.

Apart from hormonal treatments, surgery is the only treatment for endometriosis. While hormonal contraceptives, progestogens and anti-progestogens, and GnRH agonists and antagonists have all proven effective in the management of symptoms, abolition and excision of endometriotic lesions is the only remedy for endometriosis related infertility6. Studies show operational laparoscopy increases crude pregnancy rates to 57%-69% (Stage I-II) and 52%-68% (Stage III-IV), compared to just 33% (Stage I-II) and 0% (Stage III-IV) after expectant management14–16. Due to the lack of a noninvasive diagnostic test, many women with endometriosis receive suboptimal, or inappropriate care. Physicians are generally hesitant to recommend surgery for diagnostic purposes, instead administering hormonal therapies that delay diagnosis and often prevent women from receiving more effective surgical treatment. As up to 50% of cases of endometriosis are progressive, the delay in diagnosis can lead to significant increases in patient morbidity5.

**1.3 Economics of Endometriosis**

Endometriosis significantly impairs women’s quality of life, which in turn leads to encumbrances on the healthcare system. Estimates of health-related quality of life (HRQoL) in women with endometriosis show that pain, impaired psychological functioning, and reduced social functioning are the most common effects, with chronic pain and infertility imposing the greatest burden to HRQoL4. Currently the cost associated with endometriosis in surgically diagnosed Canadian patients is estimated to be $5200 annually per patient17. Extrapolating this to the estimated number of Canadian women with endometriosis amounts to a total $1.8 billion annual cost for Canadian society, an amount similar to or greater than other chronic conditions such as migraine, Crohn’s disease, diabetes, asthma and rheumatoid arthritis18. Interestingly, only 22% of these expenditures come from direct medical costs while the remaining 78% come from indirect medical costs such as lost leisure time and productivity17. As studies can only measure costs in surgically diagnosed cases of endometriosis, the true cost of the disease is impossible to ascertain and likely to be much higher due to undiagnosed cases. Furthermore, a recent review found patients with endometriosis to be at a greater risk for other chronic diseases, further decreasing quality of life and increasing health care costs for these patients19. They found patients with endometriosis to have greater risks for ovarian (in 21 studies), breast (in 14 studies), endometrial (in 8 studies) and cervical cancers (in 4 studies), as well as cutaneous melanoma (in 12 studies), and non-Hodgkin’s lymphoma (3 studies) 19. Additionally found 9 studies were found showing links between endometriosis and autoimmune diseases, 6 revealed links with asthma and atopic diseases, and 4 elucidated links with cardiovascular diseases19. As such, it is imperative to find an easy to use, non-invasive clinical marker, which will allow for earlier detection and treatment of disease. Such a test would not only improve the quality of life for women suffering with endometriosis, but also save billions of dollars in health care costs.

**1.4 Etiology of Endometriosis**

While the precise cause of endometriosis is currently unknown, several theories have been presented to help elucidate its pathogenesis. The most widely accepted of these is Samson’s theory of retrograde menstruation, which claims that endometrial tissue shed at menses flows into the peritoneal cavity and implants at ectopic locations20. While this theory would allow endometrial tissue the opportunity to adhere and proliferate, it is unlikely to be the sole cause of endometriosis, as retrograde menstruation has been shown to occur in up to 90% of women, few of whom develop the condition21. It is therefore likely that other physiological processes are affected in these women that increase their susceptibility to implantation, ultimately leading to disease. Other hypotheses of endometriosis include immune dysfunction, transplantation during surgery, genetic predisposition, Mullerian remnant differentiation, coelemic metaplasia, vascular/lymphatic transport of endometrial tissue, toxicant exposure, and oxidative stress22.

**1.5 Differential Gene Expression in Endometriosis**

A great deal of research has gone into investigating differential gene expression, leading to differential protein expression, in patients with endometriosis23. Currently over 100 different candidate genes have been identified, and shown to be differentially expressed in women with endometriosis24. While the utility of most of these genes for determining the risk of endometriosis is unknown, several have been identified that are thought to be involved in the development of disease, specifically WNT4, CDC42, HSPC157, HOX10, CDKN2BAS and FN125,26**.** In a 2015 review of genetics associated with endometriosis, Baranov et al. found candidate genes relating to 7 main disease pathways27. These were genes coding for proteins associated with sex hormones and their receptors, endometrial cell proliferation and menstrual cycling, tumor suppression and oncogenes, detoxification, polymorphic miRNA regulators, for pro-inflammatory cytokines, and angiogenesis and cell invasion27. Of particular note, they found up-regulation of genes responsible for the production of vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), interleukin 8 (IL-8), regulated on activation normal T cell expressed and secreted (RANTES), and matrix metalloproteinases 1 through 9 (MMP-1-9), all of which have been identified as putative biomarkers for the diagnosis of endometriosis1,27. Identification of differentially expressed genes has led to the identification of differentially expressed proteins and pathways in patients with endometriosis, giving clues to potential targets for treatment, and biomarkers for diagnosis.

**1.6 Pathophysiology of Endometriosis**

Regardless of the cause, central to the establishment of endometriosis is the successful adhesion, implantation, survival, and growth of exfoliated tissue3. The success of each of these processes depends on various cytokines, adhesion molecules, proteolytic enzymes, and angiogenic factors1. These proteins not only lead to endometriosis, but also stimulate other endometriotic factors highlighting a complex interplay between pathways1. It is likely that pathology in many, or all of these areas combine to allow for growth of endometriotic lesions.

**1.6.1 *Immunological***

The immune systems role in the initiation and progression of endometriosis was first noted by Dmowski in 198128. Immune response resulting in inflammation is a hallmark of endometrioses, though the mechanisms through which this occurs are poorly understood22. A pitfall in Sampson’s theory of retrograde menstruation is how the endometrial cells implant and survive at ectopic locations without being destroyed by the immune system20. The immune system of women with endometriosis seems to paradoxically favor disease progression through secretion of proteins involved with inflammation, angiogenesis, and growth29. These factors not only create the problem of chronic inflammation associated with endometriosis, but also aid in cellular adhesion, survival, invasion, proliferation, and angiogenesis29.

While macrophages, the key element of the immune system, have been shown to be significantly elevated in women with endometriosis, their phagocytic abilities were markedly decreased30,31. Instead they were found to secrete increased amounts of proteins involved in inflammation and disease pathophysiology32–34. Some of the most studied in relation to endometriosis are IL-6, IL-8, VEGF, tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN-γ), and RANTES1. MCP-1, RANTES and IL-8 are chemoattractants and are largely responsible for bringing macrophages and neutrophils to the site of endometriosis, while IL-6, TNF-α, VEGF and INF-γ are released by macrophages causing inflammation and angiogenesis33. In mouse models it has been shown that while in the absence of macrophages endometrial tissue was still able to implant and adhere to the peritoneum, angiogenic functions were lost and implants failed to grow35. This highly suggests that the involvement of macrophages and the proteins they release are requisite for the establishment of the disease.

**1.6.2 *Cellular Adhesion***

For endometrial tissue to implant at ectopic locations, molecules involved in cellular adhesion must be present31. Cells derived from endometriotic lesions have increased adhesion capacity for various components of the extracellular matrix (ECM), such as collagen type IV, laminin, vitronectin, and fibronectin compared to normal endometrium36,37. Cellular adhesion molecules are involved in cell-cell interactions and have also been implicated in inflammatory processes38. These molecules are expressed both by the epithelium of the eutopic and ectopic endometrium as well as by cells of the immune system such as macrophages38. Therefore, they may be involved not only in cellular attachment, but also in the accompanying immunological inflammatory response39.

The primary adhesion molecules indicated in endometriosis are soluble intercellular adhesion molecule 1 (sICAM-1), cadherins, and integrins, all of which have been shown to have elevated levels in the disease and are responsible for interactions necessary for the establishment of lesions38,40. Integrins are a family of cell-cell adhesion molecules, also involved in attachment to the ECM, promoting invasion36. E-cadherin is another protein important to the attachment of endometriotic lesions through binding with intracellular β-1 (IL-1β) integrins. This forms a complex with the cytoskeleton of cells essential for endometrial cells to attach to the epithelium41. sICAM-1 is a further protein found to be involved in the attachment of endometrial cells to the epithelium24. Not only is it important for attachment, but it is also involved in inhibiting natural killer cell mediated cytotoxicity, correlating with immune dysfunction24.

**1.6.3 *Implantation***

In order for endometriosis to develop, endometrial tissue must first implant at ectopic locations1. It is likely that remodeling of the ECM through proteolysis is essential, or at least contributes, to implantation of these tissues42. The two main pathways involved in ECM degradation are the plasminogen activator (PA) and matrix metalloproteinase (MMP) systems42. The PA system refers to the PAs and their conjugate inhibitors43. The PA system is associated with many processes including lysis of fibrin clots, tissue remodeling, tumour invasion, and various reproductive functions44–49. Tissue remodeling requires a fine balance between the levels of proteases and their inhibitors43. The PA system remodels tissue by converting extracellular plasminogen into plasmin, an active protease, which then degrades the ECM and activates the MMP system, which further degrades the ECM42,43. PA inhibitors have been shown to be particularly important for implantation of tissues42. SERPINE2, also known as glia-derived nexin, is one of the main serine proteases inhibitors and has been shown to be significantly overexpressed in the tissue of invasive pancreatic carcinomas43. Studies in animals and humans have additionally shown SERPINE2 to be significantly elevated during embryo implantation, possibly suggesting it may also play a roll in implantation of other tissues, such as in the case of endometriosis43.Additionally, a study of genes involved with SERPINE1, a similar protease, found that women who were heterozygous for the 4G allele, which causes increased transcription of SERPINE1, had an increased risk of endometriosis associated infertility50.

MMPs are also involved in tissue remodeling and have been implicated in implantation of endometrial tissue42. MMP-1, 2, 3, 7, and 9 have all been found to be upregulated in endometriosis, and to be induced by cytokines such as IL-1, IL-8, and TNF-α51,52. These proteases act to degrade the ECM so that endometrial implants and vasculature can grow and proliferate53.

**1.6.4 *Angiogenesis***

Angiogenesis is the physiological process through which new blood vessels form from existing vessels51. It is well established that extensive angiogenesis is required for the growth and maintenance of endometriotic lesions54. Angiogenesis is a complex process requiring the coordination of various functions before new blood vessels can ultimately be formed51. Many different proteins participate in these processes, some of which are of particular interest in relation to endometriosis.

VEGF is the most potent stimulator of angiogenesis and the most widely studied1,54*.* VEGF is estrogen and progesterone responsive, and is increased in response to cytokines, hormones, growth factors and hypoxia54. Changes in the concentration of VEGF mRNA levels occur throughout the menstrual cycle, with concentrations being highest during the secretory and menstrual phases55. Peritoneal macrophages and neutrophils have also been shown to be a potent source of VEGF55. VEGF levels are also related to concentrations of MMP-1, an interplay that could be important in the pathogenesis of the disease56.

There are also several cytokines that play a role in angiogenesis and that are thought to be related to endometriosis. IL-1β and IL-6 are produced by macrophages in patients with endometriosis and result in increased expression of VEGF57,58. IL-8 is known to be a chemoattractant for lymphocytes and neutrophils, but has also been demonstrated to stimulate angiogenesis through activation of VEGF59. Lastly, RANTES, while not directly stimulating angiogenesis, is a potent chemoattractant for macrophages, which are in turn known to secrete VEGF among other factors stimulating angiogenesis60.

Several studies on animal models have looked into the use of anti-angiogenic treatments as a remedy for endometriosis with promising results61. The use of inhibitors, resulting in decreased angiogenesis, has led to a decrease in both lesion size and vascular growth61. Furthermore, inhibition of VEGF was shown to lead to a significant decrease in the number of lesions, further indicating pathology of this pathway62.

**1.7 Putative Biomarkers of Endometriosis**

The search for clinical biomarkers has involved attempts to identify quantifiable factors differentially expressed between women with endometriosis and healthy controls. While Sampson’s theory of retrograde menstruation may be necessary for the development of endometriosis, this is known to occur in the majority of women, and is therefore likely insufficient for disease development20. It is quite likely then that dysregulation of other pathways must occur in order for women to develop this disease. Abnormalities in immune function, cellular adhesion, cellular implantation, and angiogenesis, have all been investigated in relation to endometriosis63. If these pathways are indeed affected, specific proteins involved in each may be of use to investigators as clinical biomarkers.

While over 100 putative biomarkers have already been postulated and investigated, none have proven adequately sensitive, specific, and reliable for clinical use and disease diagnosis1,3,63. While many of these have shown promise, studies often fail to control for important variables leading to inconsistent results1,63. Markers have been shown to have different concentrations for different disease locations (ovarian endometriomas vs. deep infiltrating endometriosis), for different stages of disease (I-II vs. III-IV), for different lesion types (red vs. black), and for different stages of the menstrual cycle (secretary vs. proliferative vs. menstrual) 1. Additionally, studies often fail to use consistent and representative control groups1. Failing to take into account the effects of hormonal treatment or if controls are symptomatic could lead to bias in results1. While the search for biomarkers is a key area of endometriosis research, the lack of control and consistency in past research may be a reason for the lack of success thus far5.

Another potential reason for the lack of current success in finding endometrial biomarkers is that most studies investigate only a few biomarkers at a time, often looking at only one disease pathway63. Through literature review, eight serum biomarkers, encompassing four disease pathways, have been selected for the current study. These include VEGF, IL-6, RANTES, Zn-alpha2-glycoprotein (ZAG), glycodelin, sICAM-1, leptin, and SERPINE2. I propose that through combining these biomarkers into panels to optimize sensitivity and specificity for disease diagnosis, they can be of use to aid in the early noninvasive diagnosis of endometriosis, benefiting both patients and the healthcare system.

**1.8 Endometrial Markers Included in Study**

**1.8.1 *Vascular Endothelial Growth Factor (VEGF)***

As endometrial tissue implants and proliferates new vasculature is required for its growth and survival64. VEGF is an important promoter of angiogenesis and vasculogenesis, and as such has potential as a biomarker for endometriosis64. While many studies have looked into this protein, there is a lack of consensus on its effectiveness1,63. However, this may be largely due to a lack of control for important variables, and not an indication of unreliability1.

In an extensive 2010 review, May et al found that only 2 of 5 studies investigating VEGF as a serum biomarker showed a significant difference in concentration between cases and controls1. More recently in a study comparing VEGF to the most widely studied serum biomarker, cancer antigen 125 (CA-125), in women with stage III-IV disease, VEGF in patient serum was found to have sensitivity and specificity of 93.3% and 96.7%, while CA-125 had sensitivity and specificity of 70%, 90%, respectively65. The same study also noted that following laproscopic surgery, VEGF levels dropped by 45.5%. Protein level changes in response to therapy is useful for monitoring such a therapy’s success and is a characteristic of an ideal effective biomarker65. While these results are promising, it does not indicate if such a difference would be observed in patients with earlier stage I-II disease.

VEGF was also included in 2 panels of 4 plasma markers in 2012 by Vodolazkaia et al66. This study investigated 28 putative biomarkers in the plasma of cases and controls, and created 2 biomarker panels consisting of VEGF, annexin V, CA-125, and either sICAM-1 or glycodelin66. These panels resulted in sensitivities between 81%-90% and a specificities between 63%-81% in independent training and test groups for surgically diagnosed cases, who had an additional negative preoperative ultrasound, and whose plasma samples were collected during the menstrual phase of the menstrual cycle66. Variance in accuracy in this study was due to multiple methods of analysis and the use of two separate training and test study groups66. Furthermore, using multivariate logistic regression analysis on all patients (regardless of cycle stage or whether endometriosis was ultrasound negative or not) a panel consisting of VEGF, Annexin V, and CA-125, had a sensitivity of 71% and a specificity of 67% in the training group, and a sensitivity of 85% and specificity of 75% in the test set66.

It should be noted that some studies in serum and tissue have found VEGF to be effective as a biomarker only under certain conditions1. One study found significance only during the secretory phase of the menstrual cycle, while another found it effective only for patients with red lesions, whereas black endometriotic lesions showed no increase in concentration67,68. Additionally, one study found that concentrations of VEGF were only significantly different in the tissues of patients with ovarian endometriomas, while patients with deep infiltrating endometriosis had no measurable difference when compared to controls69. Explanations for these findings include disease heterogeneity, and the greater need for angiogenesis during earlier stages of disease5.

It has also been shown that there is significantly greater concentrations of hepatocyte growth factor (HGF) in blood filled red peritoneal endometriotic lesions compared to other lesion types70,71. HGF is known to work in concert with VEGF, indicating that there may also be higher levels of VEGF in red lesions72. However this is yet to be definitively determined. While overall VEGF seems promising as a biomarker, a lack of consensus has led to its inclusion in the present study in an effort elucidate its efficacy in a novel panel of biomarkers.

**1.8.2 *Interleukin 6 (IL-6)***

It is well established that cytokines play an important role in immunity through activation, growth, and differentiation of immune cells73. Endometriosis causes local inflammation resulting in the production cytokines73. IL-6, which is a pro-inflammatory cytokine involved in T cell activation and B cell differentiation, also shows promise as a serum biomarker for endometriosis1. While many studies have shown IL-6 to be a promising marker, results have not been consistent1.Of the 12 studies investigating IL-6 as a serum biomarker for endometriosis, only 7 have had significant results74–80, while 5 have shown no link81–85. Furthermore, the accuracy of each for diagnostic purposes varies considerably1.

There are many potential reasons for the divergent results of previous studies. Many of the inconsistencies in study results may be attributed to divergent methodology, potentially effecting results. Some studies of IL-6 have looked solely at women with ovarian cysts (endometriomas versus benign cysts), while others have used inconsistent control groups consisting of either healthy controls, or controls with infertility unrelated to endometriosis biasing results78,82,83. Additionally, large variations in assay sensitivities may have affected results77,82. Lastly, disease stage inclusion criteria could have drastically affected study results, as it has been suggested that IL-6 levels may be raised in stage I-II disease but not in stage III-IV1. One study with inconclusive results excluded women with stage I endometriosis resulting in a vast majority of cases having later stage disease65. Two others that stratified cases into stage I-II and stage III-IV groups yielded sensitivities of 73% and 89.5%, and specificities 83.3% and 82.5% respectively for stage I-II disease but results for stages III-IV disease were non-significant59,61. Clearly more research into the effectiveness of IL-6 as a clinical serum biomarker accounting for possible confounding variables is needed, hence its inclusion in the current study.

**1.8.3 *Regulated on Activation, Normal T cells Expressed and Secreted (RANTES)***

The β-chemokine CCL5, also known as RANTES, is another putative cytokine biomarker showing promising but inconsistent results1. RANTES is chemotactic for T cells, basophils, and eosinophils, and is involved in recruiting leukocytes to areas of inflammation86. While many studies have reported the concentration of RANTES in peritoneal fluid, to date only 4 have investigated its potential as a biomarker in the peripheral blood86. Two studies from the same group found significantly higher concentrations of CCR1 (the RANTES receptor) mRNA in the peripheral blood of endometriosis patients but did not take any direct measure of RANTES itself87. In the only study to find significance measuring RANTES in the blood, Vodolazkaia et al. found a significant increase in patients plasma regardless of menstrual cycle in the test group, but found no such significance in the training group66. However, RANTES was not included in either of the two panels of markers proposed by the study66. Only one study to date investigating RANTES in the peripheral blood was unable to find significant results82. While some studies looking at RANTES or its receptor have shown significance, the lack of research highlights the need for its inclusion in the current study.

**1.8.4 *Zn-alpha2-glycoprotein (ZAG)***

ZAG is a glycoprotein implicated in immune response and is a known biomarker for various carcinomas88. Only one study to date has investigated ZAG as a marker for endometriosis in the peripheral blood89. Signorile and Baldi identified the protein by mass spectrometry in 2014, and confirmed differential expression in a separate cohort of patients using ELISA89. The study found that ZAG had a sensitivity of 69.4% and a specificity of 100% regardless of cycle stage or stage of endometriosis89. This is especially promising when compared to CA-125, which when analyzed in the same cohort of patients has a sensitivity of only 33%89. As these results are quite promising, and only one study has looked at ZAG as a serum biomarker, it has been included in the current study.

**1.8.5 *Glycodelin***

Glycodelin is glycoprotein recently hypothesised to play a role in endometriosis90. This endometrium derived protein is known to possess immunosuppressive and angiogenic effects, and is also believed to contribute to endometriosis and related infertility90. Moreover, glycodelin is not only produced by the endometrial epithelium, but is also shed from endometriotic lesions into peripheral blood, suggesting that increased circulating concentrations may be seen in women with the disease91.

Several studies have looked at glycodelin levels in the peripheral blood of women with endometriosis with promising results3. It was included in the aforementioned 2012 panel from Vodolazkaia et al., and was one of only 3 proteins of the 28 studied to have significance in both training and test groups regardless of menstrual cycle, along with CA-125 and leptin66. Furthermore, in the same study it was found to have the highest sensitivity and specificity of any protein during the follicular phase of the menstrual cycle66. Glycodelin was further investigated in 2013 by Kocbek et al., who found the marker to have a sensitivity of 82.1% and a specificity of 72.4% in the serum of women with ovarian endometriosis regardless of cycle phase90. Interestingly, they also found a positive correlation between glycodelin concentrations and intensity and frequency of menstrual pain, which most other putative markers have failed to do90. The same group further investigated glycodelin in conjunction with 16 other biomarkers, finding that only glycodelin had a significant difference in the serum of patients with ovarian endometriosis92. The study found the best overall performance was a model looking at the ratio of glycodelin to leptin, which had a sensitivity of 83.6% and a specificity of 83.8% when combined with age in these patients92.

To date there is only one study that has failed to find significant differences in glycodelin concentrations in women with endometriosis85. However this study used a small and unrepresentative patient population consisting of 33 cases and 17 controls ages 13-19 which may have effected results85. Again, while glycodelin certainly seems promising, more research is needed to determine its reliability as a diagnostic biomarker. The majority of current studies have solely looked at its concentration in patients with ovarian endometriomas, and its ability to identify other forms of endometriosis is largely unknown.

**1.8.6 *Soluble Intracellular Adhesion Molecule 1 (sICAM-1)***

For endometrial tissue to implant and proliferate adhesion molecules must be present38. sICAM-1 is one of the major adhesion molecules aiding in attachment of cells to ectopic locations38. It has also been shown to inhibit natural killer cell mediated cytotoxicity, potentially helping ectopic tissue evade destruction93. If natural killer cells cannot attack the foreign tissue, it could allow for the implantation and development of endometriosis93. sICAM has been shown to be continually shed from endometriotic lesions, increasing the likelihood of it entering peripheral circulation93.

Studies to date have found conflicting results, with some showing a significant increase94,95, a significant decrease66,96, or no significance97–99 when comparing concentrations of sICAM-1 between cases and controls. While some of these discrepancies may well be due to varying study designs, types of blood samples, or varying menstrual cycle phase, it may also be the case that levels of sICAM-1 change during the course of disease, first increasing in stage I-II disease, and decreasing in stage III-IV disease1,91. The two current studies finding an increase in sICAM-1 both focused on women with stage I-II disease, while one of the two showing a decrease looked exclusively at women with stage III-IV disease94–96. Vodolazkaia et al., who included sICAM-1 in their panel, found it to be significantly reduced regardless of disease stage, while another paper found it to be elevated in the serum of patients with stage III-IV disease38,66. However this latter study had a very small sample size consisting of just 5 cases and 8 controls and was not adequately powered to give meaningful results47. While certainly promising, these discrepancies again highlight the need for further research controlling for these variables24.

**1.8.7 *Leptin***

Leptin is a hormone produced in adipocytes, and is primarily responsible for the regulation of lipid metabolism, metabolic rate, and reproductive functions99. Recently it has also been found to have angiogenic, immunoregulatory, proinflammatory and mitogenic properties, and is believed to play a role in endometriosis100. Leptin can also be stimulated through inflammatory cytokines such as TNFα and IL-1100. It can in turn then stimulate proliferation of ectopic endometriotic cells, leading to growth of endometriotic lesions36,101. The majority of past studies have either found levels of leptin to be increased or to show no significant change84,101–104. Interestingly one study found baseline serum leptin levels to be unchanged in women with endometriosis, but did observe an increase in levels during treatment with danazol or leuprolide acetate, indicating that leptin may have a use for monitoring treatment101.

Two recent studies have found levels of leptin to be decreased in the blood of cases versus controls66,92. While not included in their panel, Vodolazkaia et al., found levels of leptin to be significantly decreased in their test group for all stages of the menstrual cycle66. More recently Kocbek et al. found that leptin, while not significantly decreased in their study population, could be combined with glycodelin with a resulting ratio able to differentiate cases of ovarian endometriosis from controls with a sensitivity of 83.3% and a specificity of 83.6%92.

**1.8.8 *SERPINE2 (Glia-Derived Nectin)***

While currently no studies have investigated SERPINE2 in relation to endometriosis, it has been implicated in various carcinomas such as breast, colon, gastric, prostatic, pancreatic, and testicular cancer, as well as oral squamous cell carcinoma43,105–108. Additionally it has been shown to be required for tumour growth and malignant progression43,105–108. Serpinesare expressed in tissues throughout the body and function in many processes noted in endometriosis such as tissue remodeling, inflammation and growth108,109. Along with ZAG, Signorile and Baldi found the genes for SERPINE2 to be differentially expressed in ectopic endometrial tissues regardless of menstrual stage110. As such I believe it to have promise as a biomarker for the disease.

SERPINE2 is upregulated by extracellular signal regulated kinases (ERK) signal transduction and forms complexes with its protease substrates in the ECM105. These complexes interact with the low-density lipoprotein receptor-related protein 1 (LRP1) receptor, which in turn enhances ERK signal transduction and increases expression of matrix-metalloprotease 9 (MMP-9)106. MMP-9 then cleaves SERPINE2, which enables protease-mediated remodeling of the ECM111. MMP-9 degrades the ECM allowing for growth and invasion of tissues111. As growth of endometriosis lesions highly resembles that of tumours, I believe these findings from carcinoma studies to likely be translatable to the current study.

**1.8.9 *Potential Confounders***

I believe these 8 serum proteins to be the most promising clinical biomarkers for the noninvasive detection of endometriosis. Selection was based on a combination of novelty, promise from past research, and inconsistencies from past research thought to be the result of controllable confounding variables. Past studies have often failed to control for factors with the potential to influence biomarkers efficacy, biasing results1. In this study I will take into account stage of disease, menstrual cycle stage, use of medication, patient age, BMI, smoking history, concomitant illnesses, and control symptomatology (women with surgically confirmed absence of endometriosis but with similar symptoms), all of which have been suggested as possible confounders5.

**1.9 Hypothesis**

Difficulties in the diagnosis of endometriosis have led to a considerable burden of disease, and extraneous costs to the healthcare system. Therefore the development of an easy to use clinical biomarker for the early detection of endometriosis is a priority in gynecological research and would greatly benefit all stakeholders. A good biomarker should be noninvasive, reproducible, as accurate for disease detection as possible, and show a decrease in concentration as a result of treatment3. As peripheral blood serum can be easily collected and tested for protein concentrations, it has been selected as the medium for use in the present study.

I hypothesize that serum concentrations of these eight chosen putative biomarkers will be significantly different between cases and controls, and that when combined in a panel of two or more, will have sufficient accuracy for disease detection to allow for clinical application.

**1.10 Main Study Objectives**

Endometriosis is a common chronic illness for which there is no cure and few treatment options. Furthermore, difficulties in diagnosis lead to increased morbidity, patient suffering, and to potential misdiagnosis in those with differing, but symptomatically similar conditions2. Therefore a non-invasive clinical test to diagnose endometriosis is a key area of interest among endometriosis researchers. Due to the pathology of the condition, it seems quite possible for patients suffering from endometriosis to have differing levels of clinically relevant protein markers in circulation compared to controls. While many studies have examined the use of protein biomarkers for the detection of endometriosis, none have proven fruitful to date1. The current study aims to find a valid and reliable test for the diagnosis of endometriosis though combining several biomarkers, encompassing differing disease pathways. This study hopes a panel of markers can be formed that will ultimately create a tool with sufficient accuracy for clinical application. Therefore the goals of the study are to:

1. Quantify and compare the concentrations of eight putative clinical markers of endometriosis in the serum of women with and without disease.

***Specific aims***

1. Characterize the effect of menstrual cycle, stage of disease, and hormonal treatment on circulating concentrations of each clinical marker
2. Use receiver operating characteristic (ROC) curve analysis to determine the area under the curve (AUC) for each clinical marker.
3. Determine optimal sensitivity and specificity of each marker
4. Use classification and regression trees (CART) analysis to create a panel of clinical biomarkers.

***Specific aims***

1. Use classification and regression tree analysis to compare the predictive value of different combinations of makers.
2. Create a panel of markers with sufficient accuracy to allow for clinical application.

**CHAPTER 2: MATERIALS AND METHODS**

* 1. ***Quantify and Compare the Concentrations of Eight Putative Clinical Markers of Endometriosis in the Serum of Women With and Without Disease.*2.1.1 *Study Participants***

Serum samples for this study were gathered during laproscopic surgery at McMaster University Medical Centre. 134 samples have been collected for the study. During laparoscopic surgery women were categorized as a case or symptomatic control by a gynecological surgeon with extensive experience in the diagnosis of endometriosis. Diagnosis was additionally confirmed by pathology reports. All women were asked to consent and complete a questionnaire assessing demographics, menstrual cycle length, date of last menstruation, and pelvic pain. Menstrual cycle stage was further confirmed by pathohistology. Exclusion criteria included all women unable to consent, those under the age of 18, those who were pregnant, or those with a diagnosis of adenomyosis. As adenomyosis is clinically similar to endometriosis, potential for confounding effects was avoided. After exclusion criteria the study population consisted of 96 cases and 25 controls. Participants who received hormone therapies for within at least the 3 months before study enrollment were included in the treated group of cases or controls to determine the effect of such treatment on circulating clinical markers. The case group contained 39 treated and 57 untreated patients while the control group contained 7 treated and 18 untreated patients.

An experienced surgeon determined the stage of endometriosis in cases during surgery according to the revised American Society for Reproductive Medicine (rASRM) classification system. The current study population consisted of cases with stage I disease (n=8), stage II disease (n=7), stage III disease (n=10), and stage IV disease (n=52). Disease stage information was unavailable for n=19 cases. This study was approved by the Research Ethics Board, McMaster University (REB# 12-083-T). Peripheral blood was collected from participants prior to surgery by a nurse at the McMaster University Medical Centre using plasma and serum separator tubes.

**2.1.2 *Sample Collection***

Blood samples were collected from the cubital vein into serum and plasma separator tubes by a nurse at McMaster University hospital prior to surgery. Blood was placed on ice, transferred to the laboratory, allowed to clot for 1 hour at 4°C, and centrifuged at 3,000 rpm. Approximately 200 μL of plasma or serum was aliquot into 1.8 mL cryovials (Sarstedt) and frozen at −80°C until required for analysis. In order to avoid protein degradation resulting from repeat freeze/thaw cycles, serum and plasma samples were sorted in separate aliquots and only thawed once for each assay.

**2.1.3 *Quantification of Circulating Concentrations of Clinical Markers***

Serum samples were thawed at room temperature and concentrations of each protein were quantified in duplicate using commercially available and externally validated quantitative ELISA kits following the manufacturer's protocols. The clinical markers quantified include: VEGF, IL-6, RANTES, sICAM-1 (R&D Systems [Minneapolis, Minnesota]), ZAG, Leptin (Abnova [Walnut, California]), Glycodelin (Bioserv Diagnostics [Rostock, Germany]), and SERPINE2 (Cloud-Clone Corp [Houston, Texas]). Optical densities were determined for each sample at a wavelength of 450nm, and the concentration of protein was quantified by comparison to a protein standard curve.

**2.1.4 *Detection Limits and Intra/Inter Coefficients of Variance***

The detection limits, and intra and inter assay coefficients of variation for each circulating protein measured were: VEGF (9.0pg/mL, 5.4% [intra], 7.3% [inter]), IL-6 (0.7pg/mL, 2.6% [intra], 4.5% [inter]), RANTES (2.0pg/mL, 2.4% [intra], 6.5% [inter]),

ZAG (21pg/mL, <10% [intra], <15% [inter]), glycodelin (6ng/mL, 8.3% [intra], 4.6% [inter]), sICAM-1 (0.096ng/mL, 4.6% [intra], 5.5% [inter]), leptin: (0.2ng/mL, 5.9% [intra], 5.6% [inter]), SERPINE2: (0.135ng/mL, <10% [intra], <12% [inter]).

**2.1.5 *Statistical Analysis***

Patient demographics were compared between cases and controls by t-test, Mann-Whitney rank sum test or Chi-square (SigmaStat 3.5 Systat Software Inc., Chicago, IL, USA) and are presented in **Table 1** as mean ± SD, median (25%–75% percentiles) or n (%) respectively. Results were considered statistically significant for *p*≤0.05. The concentration of VEGF, IL-6, RANTES, ZAG, glycodelin, sICAM-1, leptin and SERPINE2 were analyzed using SigmaStat 3.5. During each step of analysis outliers were determined and removed to increase accuracy of result (2xSD +/- the Mean). Circulating biomarker concentrations were compared using t-test (two groups), or one-way ANOVA (many groups) when data was normally distributed. When normality failed these tests were substituted with Mann-Whitney rank sum test and Kruskal-Wallis one-way ANOVA on ranks respectively.

To determine the effects of hormonal treatment on circulating biomarker concentrations, samples from treated and untreated controls were compared for each marker. If no significant differences were found both groups were combined to increase control group samples size. To determine the effects of menstrual cycle phase on circulating biomarker concentrations, samples from controls were additionally analyzed according to menstrual stage. If no significant differences were observed for a biomarker in either of these comparisons, subsequent analysis included all controls as one group. Comparisons were then made between all untreated cases and controls, untreated cases stratified by stage of disease (I-II vs. III-IV) and controls, and all cases stratified by treatment (treated vs. untreated) and controls for each biomarker. When statistically significant results were obtained, receiver operating characteristic (ROC) curves were generated. From ROC curves the area under the curve (AUC) was used to determine the sensitivity and specificity of each biomarker for disease diagnosis in the chosen patient population.

***2.2 Carry out CART Analysis to Create a Panel of Clinical Biomarkers***

**2.2.1 *CART Description***

Classification and Regression Trees (CART), is a decision tree software for advanced data mining developed by Sanford Systems (San Diego, CA, USA). This tool analyzes a multitude of variables that can be entered by the user to quickly reveal important data relationships that could otherwise remain hidden. Case and control concentrations of desired biomarkers are first entered into the CART database. The ideal biomarker for use as a “root node” is identified, and a cut-off value for this biomarker is given. The tree then branches, creating two additional “child nodes” based on this cut-off value. Samples containing biomarker concentrations greater than the stated cut-off value are shown on the right, and samples containing biomarker concentrations below the stated cut-off value are shown of the left. The proportion of cases and controls both above and below the cut-off value are stated in each node. The software then uses concentrations of other biomarkers included, splitting the patient population represented in the prior nodes again using determined cut-off values of the new biomarker. Depending on the number of biomarkers selected for analysis, CART will continue to split the patient population until terminal nodes are reached. Terminal nodes consist of patient populations with the highest percentage of either cases or controls. Using this software one can determine what combination of biomarkers will lead to the highest sensitivity and specificity for disease diagnosis in their study population using the cut-off values given.

**2.2.2 *Inclusion Criteria for CART Analysis***

CART analysis was used to create a panel with maximal diagnostic accuracy for the differentiation between untreated cases of endometriosis and controls. A biomarker was deemed suitable for inclusion when ROC curves resulted in an AUC≥0.6. Terminal node sample size was set at n≥3.

**CHAPTER 3: RESULTS**

***3.1 Quantify and Compare the Concentrations of Eight Putative Clinical Markers of Endometriosis in the Serum of Women With and Without Disease.***

**3.1.1 *Patient Characteristics***

All women included in this study (134) underwent laparoscopic surgery, from which 106 cases of endometriosis and 28 symptomatic controls where identified. Over all 13 women were excluded from this study owing to a diagnosis of adenomyosis (cases n=10, controls n=3). The final study population constituted 121 women: 96 cases and 25 controls (**Table 1**). Only current medication use (*p*=0.012) differed significantly between cases and control. Of the 96 confirmed cases of endometriosis, n=39 (41%) received hormonal treatment within three months prior to surgery, while n=57 (59%) were untreated. Of the 25 controls n=7 (28%) were treated while n=18 (72%) were untreated. The average age (*p*=0.72), ethnicity (*p*=0.095), occupational status (*p*=0.33), smoking status (*p*=0.50), age at first menstruation (*p*=0.25), median duration of bleeding (*p*=0.20), and menstrual cycle stage (*p*=0.62) showed no significant differences between cases and controls.

**Table 1.**

Patient characteristics of women with and without endometriosis. NSAID: non-steroidal anti-inflammatory drug, SD: standard deviation, y: years, d: days.

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristic** | **Control (25)** | **Cases (96)** | ***p* Value** |
| **Age (y), mean ± SD** | 34.3 ± 8.3 | 33.6 ± 6.5 | *p*=0.722 |
| **Stage n (%)** |  |  | NA |
|  Minimal 1 | 0(0) | 8(8) |  |
|  Mild 2 | 0(0) | 7(7) |  |
|  Moderate 3  | 0(0) | 10(11) |  |
|  Severe 4 | 0(0) | 52(54) |  |
|  Not available | 0(0) | 19(20) |  |
| **Current Med n (%)** |  |  | *p*=0.012 |
|  Hormonal contraceptives  | 4(14) | 28(27) |  |
|  Lupron | 3(11) | 21(20) |  |
|  NSAID | 2(7) | 28(27) |  |
|  Narcotic analgesic | 1(4) | 7(7) |  |
|  none/other  | 18(64) | 34(32) |  |
| **Menstrual Cycle Stage n (%)** |  |  |  |
|  Menstrual | 7(28) | 21(22) | *p*=0.6 |
|  Proliferative | 8(32) | 22(23) |  |
|  Secretory | 4(16) | 22(23) |  |
|  Unknown | 6(24) | 31(32) |  |
| **Duration of Bleeding, d Median (25%-75)** | 6(5-7) | 6(4-7) | *p*=0.198 |
| **Age at First Menstruation, y Median (25%-75)** | 13(12-14) | 12 (11-13) | *p*=0.249 |
| **Ethnicity n (%)** |  |  | *p*=0.095 |
|  Caucasian  | 19(76) | 70(73) |  |
|  Asian | 4(16) | 8(8) |  |
|  Black | 0(0) | 5(5) |  |
|  Aboriginal | 1(4) | 0 |  |
|  Unknown | 1(4) | 13(14) |  |
| **Occupational Status n (%)** |  |  | *p*=0.329 |
|  Employed | 19(76) | 54(56) |  |
|  Unemployed | 1(4) | 11(11) |  |
|  Other | 1(4) | 5(5) |  |
|  Unknown | 4(16) | 26(27) |  |
| **Smoking Status** |  |  | *p*=0.496 |
|  Yes | 5 (40) | 14 (15) |  |
|  No | 20 (80) | 77 (80) |  |
|  Unknown | 0 | 4 (4) |  |

**3.1.2 *Effect of Treatment on Peripheral Biomarkers***

To determine potential effects of treatment on circulating biomarker concentration, the treated groups of controls (n=7) were compared to the untreated group of controls (n=18). Treated controls consisted of women on hormonal contraceptives (n=4) and Lupron (n=3). Additionally while not considered a treatment in the currents study, three women in the treated control group were taking medication for pain management [NSAID’s (n=2) and narcotic analgesic (n=1)].

No significant differences were found when comparing treated and untreated controls for any of the eight biomarkers tested (***Figure 1***). Results are presented as treated controls versus untreated controls respectively. Results were obtained using a t-test when data was normally distributed or a Mann-Whitney U test when data was not normally distributed, and are presented as (Mean ± SD) or [Median (25%-75)] respectively. Mean concentrations of VEGF were found to be (214.4 pg/mL ± 167.6 vs. 288.3 pg/mL ± 216.3; *p*=0.49) **(*Figure 1a*)**. Median concentrations of IL-6 were found to be [1.41 pg/mL (0.37-1.99) vs. 0.12 pg/mL (0.001-0.87); *p*=0.091] **(*Figure 1b*)**. Median concentrations of RANTES were found to be [25703.1 pg/mL (18075.6-29921.0) vs. 43062.1 pg/mL (21415.4-681604); *p*=0.24] **(*Figure 1c*)**. Median concentrations of ZAG were found to be [53.1 pg/mL (48.3-65.3) vs. 52.5 pg/mL (9.98-68.3); *p*=0.97] **(*Figure 1d*)**. Mean concentrations of glycodelin were found to be (6.27 ng/mL ± 2.91 vs. 19.1 ng/mL ± 16.7; *p*=0.11) **(*Figure 1e*)**. Mean concentrations of sICAM-1 were found to be (11.3 ng/mL ± 2.3 vs. 10.6 ng/mL ± 2.92 4; *p*=0.55) **(*Figure 1f*)**. Median concentrations of leptin were found to be [45.9 ng/mL (31.5-109.9) vs. 40.6 ng/mL (24.4-64.8); *p*=0.34] **(*Figure 1g*)**. Median concentrations of SERPINE2 were found to be [14.8 ng/mL (11.9-43.0) vs. 15.6 ng/mL (13.6-20.1); *p*=1.00] **(*Figure 1h*)**.















***Figure 1.***

***Figure 1.*** Concentrations of putative biomarkers **(A-H)** were compared between controls who had received hormonal treatment within three months prior to study enrollment **(CoH)** and those who had not received such treatment **(CoNH).** This was conductedto determine the effects of hormonal treatment on participants lacking endometriosis**.** No significant differences were observed showing that hormonal treatment did not significantly effect circulating biomarker concentrations in patients lacking endometriosis in our study population.

**3.1.3 *Effect of Menstrual Cycle Phase on Biomarker Concentrations***

To determine variation in circulating biomarker concentration over the menstrual cycle, concentrations in control samples collected from the menstrual (n=7), secretory (n=4), and proliferative (n=8) phases were compared using one-way ANOVA (mean ± SD) or Kruskal-Wallis one-way ANOVA on ranks [median (25-75%)]. Data on menstrual stage was unavailable for n=6 controls.

No significant differences in concentrations of any putative biomarker were found over the course of the menstrual cycle **(*Figure 2*)**. Results are presented as the menstrual phase versus secretory phase versus proliferative phase respectively. Median concentrations of VEGF were found to be [248.7 pg/mL (116.2-384.0) vs. 58.1 pg/mL (6.69-109.5) vs. 361.7 pg/mL (96.6-785.3); *p*=0.15] (***Figure 2a***). Median concentrations of IL-6 were found to be [0.37 pg/mL (0.07-1.49) vs. 0.31 pg/mL (0.12-0.49) vs. 0.56 pg/mL (0.002-2.16); *p*=0.92] (***Figure 2b***). Mean concentrations of RANTES were found to be (39109.0 pg/mL ± 20732.0 vs. 63446.8 pg/mL ± 28072.0 vs. 39096.0 pg/mL ± 24820.3; *p*=0.27) (***Figure 2c***). Mean concentrations of ZAG in controls were found to be (53.3 pg/mL ± 13.0 vs. 76.6 pg/mL ± 44.8 vs. 52.3 pg/mL ± 19.4; *p*=0.26) (***Figure 2d***). Median concentrations of glycodelin were found to be [10.5 ng/mL (4.97-37.3) vs. 14.2 ng/mL (3.58-193.7) vs. 8.63 ng/mL (6.43-17.7); *p*=0.98] (***Figure 2e***). Mean concentrations of sICAM-1 were found to be (223.3 ng/mL ± 51.4 vs. 187.6 ng/mL ± 48.1 vs. 210.1 ng/mL ± 41.1; *p*=0.49) (***Figure 2f***). Mean concentrations of leptin were found to be (63.0 ng/mL ± 49.7 vs. 34.5 ng/mL ± 18.5 vs. 48.8 ng/mL ± 26.6; *p*=0.39) (***Figure 2g***). Median concentrations of SERPINE2 were found to be [19.1 ng/mL (11.9-28.04 vs. 20.7 ng/mL (14.3-25.8) vs. 15.2 ng/mL (14.6-20.8); *p*=0.94 (***Figure 2h***).









***Figure 2.***









***Figure 2.*** Concentrations of putative biomarkers **(A-H)** were compared between all controls based on menstrual cycle phase at the type of sample collection (**M**: menstrual, **S**: secretory, **P**: proliferative). No significant differences were observed showing that menstrual cycle phase did not effect circulating biomarker concentrations in study participants without endometriosis in our study population.

**3.1.4 *Biomarkers and Endometriosis***

To determine the effect of endometriosis in circulating biomarker concentrations, putative markers were compared in samples from untreated cases (n=57) and all controls (n=25) (***Figure 3***). Results were obtained though t-tests when data was normally distributed and presented as (Mean+/-SD). When normality could not be achieved Mann-Whitney U tests were used with data presented as [Median (25%-75%)]. Results are presented as untreated cases versus controls respectively.

Median circulating concentrations of VEGF in cases vs. controls were [212.8 pg/mL (124.1-339.2) vs. 269.64 pg/mL (96.58-350.08); *p*=0.98] (***Figure 3a***). Median concentrations of IL-6 were found to be [0.1 pg/mL (0.06-0.8) vs. 0.35 pg/mL (0.061-1.41); *p*=0.58] (***Figure 3b***). Median concentrations of RANTES were found to be [37,357.4 pg/mL (19,887.5-65,460.6) vs. 33,356.7 pg/mL (21,415.4-62,117.0); *p*=0.57] (***Figure 3c***). Median concentrations of ZAG were found to be [73.7 pg/mL (46.6-115.3) vs. 50.4 pg/mL (41.7-64.9); *p*=0.009] (***Figure 3d***). Median concentrations of glycodelin were found to be [47.1 ng/mL (21.6-92.2) vs. 12.3 ng/mL (5.2-31.4); *p*<0.001] (***Figure 3e***). Mean concentrations of sICAM-1 were found to be (218.1 ng/mL ± 51.0 vs. 208.4 ng/mL ± 46.0; *p*=0.44 (***Figure 3f***). Median concentrations of leptin were found to be [16.4 ng/mL (8.3-25.2) vs. 13.5 ng/mL (8.7-22.7); *p*=0.81] (***Figure 3g***). Median concentrations of SERPINE2 were found to be [17.0 ng/mL (14.3-20.7) vs. 16.1 ng/mL (13.4-24.8); *p*=0.81] (***Figure 3h***).

ROC curves were generated for each putative biomarker (***Figure 4***). Only glycodelin and ZAG showed a significant difference between untreated cases and controls. This resulted in an AUC of 0.80 (*p*<0.001) for glycodelin (***Figure 4e***), and 0.69 (*p*=0.008) for ZAG (***Figure 4d***). The sensitivity and specificity of each marker was then determined at cut-off values chosen to maximize marker accuracy. At a cut-off value of 19.8 ng/mL glycodelin achieved a sensitivity of 81.6% (CI 65.7-92.3) and a specificity of 69.6% (CI 47.1-86.8). At a cut-off value of 91.6 pg/mL ZAG achieved a sensitivity of 46% (CI 31.8-60.7) and a specificity of 100% (CI 85.2-100).











***Figure 3.***







***Figure 3.*** Circulating concentrations of putative biomarkers **(A-H)** were compared between cases who had received no hormonal treatment for at least three months prior to study enrollment (**CaNH**) and all controls (**Co**). Concentrations of both ZAG (*p*=0.009) (**D**) and glycodelin (*p*<0.001) (**E**) were found to be significantly higher in untreated cases (**CaNH**) than in controls (**Co**) No significant differences were observed between groups for the remaining biomarkers.



***Figure 4.***







 





***Figure 4.*** ROC curves were generated for each putative biomarker **(A-H)** with the AUC denoted as “A” shown on each graph.Only ZAG (**D**), and glycodelin (**E**) showed significant results with respective AUC values of 0.69 (*p*=0.008) and 0.80 (*p*<0.001) outlined in red.

**3.1.5 *Biomarkers and Stage of Disease***

The effect of disease stage on peripheral biomarker was determined through comparing biomarker concentrations of untreated cases with stage I-II disease (n=8), untreated cases with stage III-IV disease (n=44), and controls (n=25). Analyses were conducted using either one-way ANOVA if data was normally distributed and presented as (Mean ± SD). If data was not normally distributed analysis was conducted using Kruskal-Wallis one-way ANOVA on ranks and presented as [Median (25%-75%)]. Data is presented as stage I-II versus stage III-IV versus controls respectively.

Median circulating concentrations of VEGF were found to be [229.1 pg/mL (115.0-336.9) vs. 214.4 pg/mL (126.9-342.6) vs. 269.6 pg/mL (96.6-350.1); *p*=1.00] (***Figure 5a***). Median concentrations of IL-6 were found to be [0.14 pg/ml (0.05 -1.69) vs. 0.08 pg/mL (0.007-0.58) vs. 0.34 pg/mL (0.06-1.41); *p*=0.42] (***Figure 5b***). Median concentrations of RANTES were found to be [24,958.0 pg/mL (13,832.2-44,347.1) vs. 42,101.4 pg/mL (25,386.0-70,285.5) vs. 33,356.7 pg/mL (21,415.4-62,117.0); *p*=0.24] (***Figure 5c***). Median concentrations of ZAG were found to be [84.7 pg/mL (48.1-121.4) vs. 72.6 pg/mL (45.4-119.5) vs. 52.5 pg/mL (42.1-66.3); *p*=0.067] (***Figure 5d***). Median concentrations of glycodelin were found to be [21.6 ng/mL (10.7-29.8) vs. 61.5 ng/mL (26.8-123.6) vs. 12.3 ng/mL (5.19-31.4); *p*<0.001] (***Figure 5e***). Mean concentrations of sICAM-1 were found to be (198.14 ng/mL ± 64.78 vs. 218.97ng/mL ± 44.32 vs. 208.44ng/mL ± 45.99; *p*=0.45) (***Figure 5f***). Median concentrations of leptin were found to be [16.7 ng/mL (3.76-27.3) vs. 16.4 ng/mL (10.8-25.2) vs. 13.4 ng/mL (8.5-21.1); *p*=0.77] (***Figure 5g***). Median concentrations of SERPINE2 were found to be [15.8 ng/mL (6.88-23.0) vs. 16.6 ng/mL (14.3-18.9) vs. 16.1 ng/mL (13.4-24.8); *p*=0.84 (***Figure 5h***).

Glycodelin was the only biomarker to show significant variation in concentration between stages of disease (**Figure 5e**). Median glycodelin concentrations were found to be significantly greater in stage III-IV disease than in stage I-II disease or controls. No such significance was found between stage I-II disease and controls. When reanalyzed using a Mann-Whitney *U* test, median glycodelin concentrations in stage I-II versus stage III-IV disease [21.6 ng/mL (10.7-29.8) vs. 61.5 ng/mL (26.8-123.6); *p*=0.027], were found to be significantly elevated in later stage disease. No statistical difference in median glycodelin concentrations was observed between women with stage I-II disease versus the control group [21.6 ng/mL (10.7-29.8) vs. 12.3ng/mL (5.19-31.4) *p*=0.41]. While these results should be noted, the small sample size of women with stage I-II disease (n=8) does not provide adequate power for meaningful results.

***Figure 5.***

  





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 b

 a

a

***Figure 5.*** Concentrations of putative biomarkers **(A-H)** were compared between untreated cases with stage I-II endometriosis (**Ca1-2**) untreated cases with stage III-IV endometriosis (**Ca3-4**) and all controls (**Co**). Only concentrations of glycodelin (*p*<0.001) (**E**) were found to be significantly different between groups, with **Ca3-4** being significantly greater than either **Ca1-2** or **Co**. No significant difference in glycodelin concentrations was observed between **Ca1-2** vs. **Co**. Bars with differing letters are significantly different.

**3.1.6 *Biomarkers and Hormonal Treatment***

The effect of hormonal treatment on circulating levels of putative biomarkers in patients with endometriosis was assessed (***Figure 6***). The treated group (n=39) consisted of cases with stage I-II (7/39) and stage III-IV (18/39) disease. Treatments included oral contraceptives (34/39) and Lupron (19/39). Disease stage was undetermined for n=14 treated patients. Women in the untreated group (n=57) received no treatment for endometriosis (36/57), or were using NSAIDs (21/57), or narcotic analgesics (4/57) for pain management. The untreated group consisted of women in stage I-II (8/57) and stage III-IV (44/57) disease. Disease stage was undetermined in n=5 untreated cases. Results are presented as treated cases versus untreated cases versus controls respectively.

Median circulating concentrations of VEGF were found to be [270.7 pg/mL (135.2-439.4) vs. 214.4 pg/mL (124.0-340.3) vs. 269.6 pg/mL (96.6-350.2); *p*=0.36] (***Figure 6a***). Median concentrations of IL-6 were found to be [0.11 pg/ml (0.06-0.89) vs. 0.1 pg/mL (0.05-0.80) vs. 0.34 pg/mL (0.06-0.41); *p*=0.85] (***Figure 6b***). Median concentrations of RANTES were determined to be [34,634.7 pg/mL (20,509.4- 56,123.6) vs. 37,357.4 pg/mL (19,887.5-65,490.6) vs. 33,356.7 pg/mL (21,415.4-62,117.0); *p*=0.85] (***Figure 6c***). Median concentrations of ZAG were found to be [74.7 pg/mL (55.1-110.5) vs. 73.7 pg/mL (46.6-115.3) vs. 50.4 pg/mL (41.7-64.9); *p*=0.009] (***Figure 6d***). Median concentrations of glycodelin were found to be [8.37 ng/mL (4.78-15.9) vs. 47.1 ng/mL (21.6-92.2) vs. 12.3 ng/mL (5.19-31.4); *p*=<0.001] (***Figure 6e***). Mean concentrations of sICAM-1 were found to be (221.1 ng/mL ± 57.5 vs. 215.5 ng/mL ± 48.2 vs. 208.4 ng/mL ± 46.0; *p*=0.66) (***Figure 6f***). Median concentrations of leptin were found to be [12.5 ng/mL (7.65-22.6) vs. 15.9 ng/mL (8.16-22.8) vs. 13.4 ng/mL (8.5-21.1); *p*=0.80] (***Figure 6g***). Median concentrations on SERPINE2 were found to be [17.5 ng/mL (15.2-20.8) vs. 16.6 ng/mL (14.0-19.6) vs. 15.5 ng/mL (13.06-22.18); *p*=0.40] (***Figure 6h***).

Of the eight markers quantified, only glycodelin (*p*<0.001) and ZAG (*p*=0.009) demonstrated a significant difference when treated cases were compared to untreated cases and controls. As previously identified, glycodelin showed a significant difference between untreated cases and controls (*p*<0.001). However, no such significance was found when treated cases where compared to controls using a Mann-Whitney *U* test [8.37 ng/mL (4.78-15.9) vs. 12.3 ng/mL (5.19-31.4); *p*=0.49]. Additionally, median glycodelin concentrations were significantly lower in treated cases than untreated cases [8.37 ng/mL (4.78-15.9) vs. 47.1 ng/mL (21.6-92.2); *p*<0.001]. These findings indicate that glycodelin concentrations decline in response to treatment. A predictable change in a biomarkers concentration as a result of treatment is valuable, as it can give physicians an easily measurable tool to track treatment success, or the reoccurrence of disease.

Using the same statistical method, median concentrations of ZAG for treated cases, untreated cases, and controls were found to be [74.7 pg/mL (55.1-110.5) vs. 73.7 pg/mL (46.56-115.3) vs. 50.4 pg/mL (41.7-64.9); *p*=0.009](***Figure 6e***). Significant differences in ZAG concentrations were observed both when comparing treated cases to controls (*p*=0.003) and when comparing untreated cases and controls (*p*=0.009). However, there was no significant difference in ZAG concentrations observed when comparing treated cases to untreated cases (*p*=0.996). These results indicate that serum ZAG concentrations are unaffected in response to treatment. While concentrations of an ideal biomarker should lessen in response to treatment, a marker without this property could have clinical relevance as all patients could be tested equally regardless of medication history.

***Figure 6.***



   

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a

 b

b

 a

 a

  

***Figure 6.*** Concentrations of putative biomarkers **(A-H)** were compared between treated cases (**CaH**), untreated cases (**CaNH**), and all controls (**Co**). Concentrations of ZAG (*p*=0.009) (**D**) and glycodelin (*p*<0.001) (**E**) were found to be significantly different between groups. Concentrations of ZAG were significantly different between **CaH** vs. **Co**, and **CaNH** vs. **Co**, but not between **CaH** vs. **CaNH**. Concentrations of glycodelin were significantly different between **CaNH** vs. **Co**, and **CaH** vs. **CaNH**, but not between **CaH** vs. **Co**. Bars with differing letters are significantly different.

***3.2 Carry out CART Analysis to Create a Panel of Clinical Biomarkers***

Any marker with an ROC value greater than or equal to 0.60 was included in analysis using CART software (Salford Systems). The only two markers investigated able to satisfy this criterion were glycodelin (AUC=0.80), and ZAG (AUC=0.60). When analyzed independently these markers were found to have a sensitivities and specificities 81.6% and 69.6%, and 46% and 100% respectively. When combined using CART however they were found to be able to diagnose disease with a sensitivity of 90% and a specificity of 65% (***Figure 7***).

This was found using an initial cut-off value of 91.6 pg/mL, the same value determined in independent analysis. 100% of samples with concentrations below this cut-off value were correctly identified as controls (specificity), while 46% of samples with concentrations above this cut-off value were correctly identified as cases (sensitivity). Glycodelin was then used as the biomarker in the subset of samples with ZAG concentrations below 91.6 pg/mL (68% of the study population). In this subset of patients, a glycodelin cut-off value of 16.0 ng/mL was able to correctly identify 81.5% of cases (samples above this value) and 65% of controls (samples below this value), giving it a sensitivity of 81.5% and a specificity of 65% for this subdivision of samples. When used in conjunction these two biomarkers had a sensitivity of 90% and a specificity of 65% for disease diagnosis.

***Figure 7.***



**Figure 7.** The class assignment of subjects in each node is shown under the node number. Class 0 is the control group, and class 1 is the endometriosis group. Bars give a graphical representation of the proportion of subjects from each group assigned to that child node. Splitting variables are shown in the parent node, with the cutoff for the split shown above the child node in gray*.* N = number of subjects.

**CHAPTER 4: DISCUSSION**

**4.1 Summary of Findings**

The present study quantified the circulating concentrations of eight putative clinical biomarkers in patients with surgically, and histologically confirmed endometriosis (n=96), and compared these concentrations to those found in symptomatic controls (n=25). Findings from this study show that serum concentrations of two of the eight studied biomarkers, glycodelin and ZAG, were significantly elevated in untreated cases versus controls. Using Mann-Whitney *U* test, differences in median concentrations (25%-75%) of glycodelin where found to be [47.1 ng/mL (21.6-92.2) vs. 12.3 ng/mL (5.19-31.4); *p*<0.001], while median concentrations of ZAG where found to be [73.7 pg/mL (46.6-115.3) vs. 50.4 pg/mL (41.7-64.9); *p*=0.009] in cases and controls respectively. Glycodelin additionally showed a significantly lower concentration in treated versus untreated cases (*p*<0.001). When determining maximal overall accuracy, glycodelin showed a sensitivity and specificity of 81.6% and 69.6% respectively, while ZAG showed a sensitivity of and specificity of 46% and 100% respectively. Results of the present study show that independently, glycodelin and ZAG have value as “rule-out” and “rule-in” triage markers to help guide physicians in determining if patients should go on to receive a more definitive diagnosis through surgery. However when combined as a panel, the sensitivity and specificity for these markers was found to be 90% and 65% respectively, showing an accuracy greater than either marker alone. No other markers tested were found to have value for the diagnosis of endometriosis.

**4.2 Relation to Existing Literature**

**4.2.1 *Glycodelin***

Results of this study are generally consistent with those of past literature. In 2013 Kocbek et al. found glycodelin to be significantly elevated in the serum of women with ovarian endometriosis (n=57) compared to controls (n=42) [mean ± SEM: (33.48 ± 6.03 ng/mL versus 8.46 ± 1.76 ng/mL)]90. At a cut-off value of 2.07 ng/mL they found that glycodelin, combined with age and BMI, had a sensitivity of 82.1% (CI 70-91) and specificity of 78.4% (CI 63-90) in their study population90. Additionally, in 2014 using an overlapping study population [ovarian endometriosis (n=58), controls (n=40)] the same group of researchers found serum glycodelin to have a sensitivity of 81.8% and a specificity of 59.5% at a cut-off of 4.7 ng/mL92.

In 2012 Vodolazkaia et al. found glycodelin to be significantly elevated in the plasma of women with laparoscopically diagnosed endometriosis who additionally had no evidence of endometriosis on a preoperative gynecological ultra-sound (n=175), compared to surgically confirmed controls (n=121)66. This study additionally separated patients into independent training and tests sets for validation66. The training set consisted of 117 cases and 81 controls while the test set consisted of 58 cases and 40 controls66. Median concentrations of glycodelin were found to be significantly elevated in cases versus controls in both training [31 ng/mL (13-53) vs. 14 ng/mL (8-31); *p*=0.002] and test [37.4 ng/mL (12.9-79.8) vs. 22.4 ng/mL (6.8-32.4); *p*=0.03] data sets66. Using ROC analysis they determined an optimal cut-off value of 18 ng/mL66. This resulted in a sensitivity and specificity of 66% and 61% in the training set, and 62% and 43% in the test set respectively66. Additionally, through subset analysis they found the greatest difference in glycodelin concentration of ultrasound negative cases versus controls to occur in the training set, when only patients with samples collected during the follicular phase of the menstrual cycle were included [24 ng/mL (8.5-45.4) vs. 8.60 ng/mL (4.5-12.7); *p*=0.009]66. However, no significance was found in the test set for this patient population so the results are not necessarily meaningful66. ROC analysis of this subset of patients showed that at a cut-off value of 9.0 ng/mL sensitivities of 74% and 70%, and specificities of 57% and 36% in respective training and test groups could be achieved66.

Our finding of glycodelin having a sensitivity of 81.6% and a specificity of 69.6% are similar those of Kocbek et. al, who found respective values of 82.1% and 78.4%, and 81.8% and 59.5% in two overlapping populations90,92. However the cut-off values that achieved these results (2.07 ng/mL, and 4.7 ng/mL) are much lower than our finding of 19.8 ng/mL. This discrepancy may be attributable to study inclusion criteria. The patient populations used in these two studies included both hormonally treated and untreated cases, without any analysis to rule out confounding effects as a result of treatment90,92. As concentrations of an ideal biomarker should move in the direction of the control group in response to treatment, a washout period of at least three months is recommended prior to analysis1. The current study found glycodelin concentrations in cases who had been hormonally treated (n=39) to be significantly lower than in those who were untreated (n=57), [9.27ng/mL (4.82-19.3) vs. 47.1ng/mL (21.6-92.2); *p*<0.001], respectively. We additionally found glycodelin concentrations to be non-significantly lower in treated cases than in controls [9.27ng/mL (4.82-19.3) vs. 12.3ng/mL (5.19-31.4)]. The failure of Kocbek et. al to control for the use of hormonal therapies may therefore be the reason for their lower cut-off value, and it would be reasonable to think that if such cases were removed, the accuracy of glycodelin as a biomarker would increase in their study population.

In contrast, while results from Vodolazkaia et al. suggest a similar cut-off value as the current study (18 ng/mL vs. 19.18 ng/mL), the accuracy of glycodelin as a biomarker was markedly decreased. They found sensitivities of 66% and 62%, and specificities of 61% and 43% in independent training and test populations, suggesting a much lower diagnostic value than the current study66. This discrepancy may also be attributable to divergent study populations. Firstly, only cases who had previously received a negative preoperative ultrasound result were included in their analysis66. As more severe cases would be more likely to have lesions visible via ultrasound, their resultant study population largely consisted of patients with early stage disease66. Their training group consisted of 99 untreated cases with stage I-II disease, and only 18 untreated cases with stage III-IV disease. Similarly their test group contained 47 patients with stage I-II disease and only 11 with stage III-IV disease. In contrast, untreated cases in the current study population consisted of only 8 women with stage I-II disease and 44 women with stage III-IV disease. The prospective nature of this study made it difficult to obtain samples from patients with early stage disease, as these patients are less likely to receive surgical therapy. These divergent study populations can be both beneficial and detrimental in a clinical setting. It could be argued that our study populating consisting largely of women with later stage disease is more clinically relevant, as patients with stage III-IV disease are more likely to seek medical attention, and thus require a diagnostic test. However as endometriosis is particularly hard to detect in early stage disease, a marker capable of detecting stage I-II disease with relative accuracy could be particularly useful. This could result in quicker implementation of treatment, decreased patient morbidity, and less healthcare burden. Future studies on the use of glycodelin as a biomarker for endometriosis should ensure an even distribution of disease stage in their study population.

The present study is the first to show a significant elevation of circulating glycodelin in women with endometriosis regardless of type, stage, BMI, age or cycle phase. Additionally, through the inclusion of a treated group of cases, we were able to identify biomarker response to hormonal treatment. Differences in biomarker concentration throughout the menstrual cycle were additionally analyzed, though no significant difference was found. However past work has found serum glycodelin concentrations to be lowest during ovulation, then to rise during the secretory phase, and peak during the early proliferative phase of the next menstrual cycle90,92. While we observed no such finding, this may be due to the small sample size available in the control group analyzed (menstrual n=7, secretory n=4, proliferative n=8). Future research should ensure menstrual cycle phase is properly controlled for.

Future studies of glycodelin must also ensure standardization of case and control inclusion criteria. The current study chose to include all cases of surgically and histologically confirmed disease, while Kocbek et al. and Vodolazkaia et al. looked only at cases of ovarian endometriosis, and ultrasound negative endometriosis respectively66,90,92. Additionally, the use of only ultrasound negative cases led to a patient population with a much higher proportion of early stage disease than in most published literature63. Lastly, as we found hormonal contraceptive use to lower circulating glycodelin concentrations, it is recommended that future studies include both treated and untreated patients to confirm our findings. This was not conducted in either paper by Kocbek et al. decreasing the validity of their findings90,92.

As the study by Vodolazkaia et al. obtained samples from a pre-existing biobank, they had access to a much larger sample size than the current study66. However as they did not collect and catalogue the samples themselves it leaves room for bias. The current study found cases with stage I-II disease to have significantly less circulating glycodelin than those with stage III-IV disease. In concert with the findings of Vodolazkaia et al., whose patient population predominantly consisted of cases with early stage disease and whose results showed comparatively lower diagnostic accuracy, it suggests that glycodelin concentrations may increase as the disease progresses. If this is in fact correct, then the accuracy of glycodelin as a biomarker for endometriosis as presented by Vodolazkaia et al. is underrepresented. Furthermore, pathology results confirming disease diagnosis was absent in 12.9% of cases included in their analysis66. This may have resulted in misclassification bias, particularly as earlier stage disease is harder for physicians to correctly identify63. This could mean that some patients analyzed as cases actually lacked the disease. This would push their results towards the null hypothesis, providing further explanation for their findings of lower diagnostic accuracy than the current study.

Only one study to date has not found a significant elevation in circulating glycodelin concentration in women with endometriosis85. In 2012, Drosdzol-Cop et al. investigated glycodelin concentrations in 50 adolescent girls aged 13-1985. Diagnosis was surgically confirmed by laparoscopy in 33 patients, while no evidence of disease was seen in 17 patients85. While no significant difference in glycodelin concentration was found in this study, the young age group selected, small sample size, and failure to exclude hormonally treated cases may be the reason for the negative results of this study85.

**4.2.2 *ZAG***

Our study found a significant elevation in circulating concentrations of ZAG in patients with endometriosis compared to controls (*p*=0.009), and is only the second study to investigate ZAG as a potential biomarker for endometriosis89. It is additionally the only study using blood serum as a medium, and to use symptomatic controls, which eliminates misclassification bias. Through ROC analysis the present study found ZAG to have an AUC of 0.69 (*p*=0.008), resulting in a sensitivity of 46%, and a specificity of 100% for disease detection.

This finding is similar to that of the previous study conducted by Signorile and Baldi89. They first identified ZAG as a putative biomarker of endometriosis through proteomics, when differential expression was observed when comparing five women with endometriosis to five controls89. They then recruited an additional 120 cases, and 20 controls (healthy women) resulting in a study population of n=125 (cases) and n=25 (controls)89. All women who had taken hormonal contraceptives within six months of study enrollment were excluded from their analysis89. In this patient population they found plasma concentrations of ZAG to be significantly elevated in cases compared to controls (*p*=0.019)89. However, the precise concentration was undisclosed. Using ROC analysis they determined ZAG to have a sensitivity of 69.4% and a specificity of 100% for disease diagnosis, though a cut-off value was unspecified89.

The results from Signorile and Baldi are consistent with those of the current study, which showed ZAG to be significantly elevated in untreated cases versus controls [73.7 pg/mL (46.6-115.3) vs. 50.4 pg/mL (41.7-64.9); *p*=0.009], respectively. This yielded a sensitivity of 46% (CI 31.8-60.7) and specificity of 100% (CI 85.2-100), similar to that of Signorile and Baldi. The current study also improved several limitations observed in the work by Signorile and Baldi. Firstly, their study made no effort to control for potentially confounding variables89. While they did state that the initial five endometriosis patients used for proteomics were confirmed through surgery and histology, no such statement was made for the additional 120 cases used for analysis89. Additionally, the only inclusion criterion for controls was women who were regularly cycling, leading to possible misclassification bias89. They also provided no information on patient characteristics, and made no effort to control for menstrual cycle phase or stage of disease89. While the current study found no difference in any patient characteristic analyzed, it is still essential for proper methodology. Both the current, and past study found ZAG to have higher specificity than sensitivity. As these are the only two studies that have investigated the use of ZAG as a biomarker for endometriosis, and they both yielded similar findings, ZAG’s potential for clinical application is certainly promising. However more studies replicating these findings are needed for further validation.

Insignificant results from the remaining six biomarkers investigated in this study are also in accordance with the literature. Twelve prior studies have investigated the use of IL-6 as a biomarker for endometriosis, with five failing to find significance and the results of the remaining seven exhibiting great heterogenaity74-85. Of the studies showing significance, sensitivities ranged from 20%-89%, while specificities ranged from 66%-80%74-80. Additionally cut-off values ranged from 1.03 pg/mL to 25.75 pg/mL74-80. Due to these vast discrepancies, the lack of significant results in the current study is not surprising. The results obtained for RANTES were again unsurprising. Only two prior studies, one consisting of independent test and training groups, have investigated its potential as an endometrial biomarker66,82. Of these three study populations, only one found RANTES to be significantly elevated in endometriosis patients66. As two thirds of the patient populations investigating RANTES as a peripheral biomarker did not show significant differences in concentrations, our findings were not surprising. Previous results for sICAM-1 as an endometrial biomarker vary considerably, with some showing a significant increase94,95, a significant decrease66,96, or no significance at all97–99. While it has been hypothesized that sICAM-1 concentrations increase in early stage disease when tissue is initially implanting, and then decrease in later stage disease as tissue undergoes apoptosis, this was not observed in the current study when cases were stratified by stage of disease96. Leptin is another putative biomarker with extremely inconsistent results. Prior studies have found leptin to be significantly increased84, significantly decreased66,92, or to show no significant change at all101-104. Current findings of non-significant differences in peripheral concentration between cases and controls are thus consistent with the bulk of the literature. As this was the first study to look at SERPINE2 as a biomarker for endometriosis, our findings suggest it is likely ineffective for this purpose.

While genes for SERPINE2 have been found to be differentially expressed in ectopic endometrial tissue, this does not necessitate the proteins presence in peripheral circulation110.

**4.3 Diagnostic Tests of Endometriosis**

We propose that an ideal clinical marker of endometriosis should be measureable in blood, sensitive and specific in identifying patients with all stages of the disease, and decrease in response to medical and surgical therapies. In addressing this last point, only glycodelin showed a significant decrease in treated versus untreated cases (*p*<0.001). If treatment decreases the prevalence or severity of a disease, and biomarker concentrations change at a proportional rate, then the biomarker is likely involved in some step of pathogenesis. While alternatively glycodelin concentration may be directly affected by contraceptive use without involvement in endometriosis, this is still a finding of note as very few putative biomarkers to date have been shown to have circulating concentrations that move toward those of healthy women in response to treatment63.

In determining the value of a biomarker, a ROC curve is a valuable tool able to discriminate individuals with and without disease. From this, the area under the curve (AUC) determines a markers ability to discriminate disease. For example an AUC of 1 would mean the marker could perfectly identify disease, while an AUC of 0.5 would mean it could identify disease status only 50% of the time. Based on previously published literature, the clinical value of a laboratory test with AUC values between 0-0.5, 0.5-0.7, 0.7-0.9 or >0.9 can be defined as zero, limited, moderate, and high, respectively63. Based on this the current study sought to identify markers with at least moderate diagnostic potential. Only two of eight markers met this criteria being glycodelin (AUC=0.80) and ZAG (AUC=0.69). ROC analysis additionally identifies cut-off concentrations resulting in varying sensitivities and specificities of the biomarker in question depending on the chosen value. While an ideal test would perfectly identify all cases and exclude all controls, having both a sensitivity and specificity of 1, this is rarely possible and most tests have either a higher sensitivity and lower specificity or vice versa. A test with high sensitivity indicates that there are a low number of patients with a negative test who actually have the disease (i.e. a low number of false negatives), while a test with high specificity means that of the patients with a positive test result, a low number do not have the disease (i.e. low false positives). In the case of biomarkers with concentrations known to be higher in cases than controls, a lower cut-off value will result in proportionally higher sensitivity than specificity (as fewer patients will be classified as controls) while a greater cut-off value will have the opposite effect (as fewer patients will be classified as cases). As typically an increase in one value corresponds to a decrease in the other, it is important to select a cut-off value ideal for the intended test purpose. If the goal of a novel diagnostic test is to replace the current gold standard, this novel test must have either increased accuracy, or similar accuracy with other advantages (expediency, cost/benefit, etc.) to be clinically applicable.

In the case of diagnosing endometriosis, the current gold standard is laparoscopic surgery. To date only one systematic review has looked at the accuracy of this test, finding it to have a sensitivity of 95%, and a specificity of 79%112. Therefore the current study, corroborated by findings in the 2016 Cochrane review on biomarkers for the diagnosis of endometriosis, suggests any new test aiming to replace laparoscopy as a diagnostic tool should show similar or greater diagnostic accuracy63. The closest this study came to achieving this accuracy in a single biomarker came at cut-off value of 9.58 ng/mL for glycodelin (sensitivity= 97%, specificity=43%). Additionally, when used in a panel with ZAG, the two biomarkers could differentiate disease with a sensitivity of 90% and a specificity of 65%. As no putative biomarker, or panel of biomarkers to date have shown levels of accuracy similar to that of laparoscopy, we cannot be disappointed with our findings.

**4.4 Biomarkers as Triage Tests**

As opposed to a replacement test, a triage test is used as an initial step to determine what patients should go on for further testing using the current gold standard, in this case laparoscopy. A triage test does not aim to replace or increase the accuracy of an existing test, but rather reduce the number of individuals who receive a diagnostic test unnecessarily. As laparoscopy is an invasive procedure, a triage test able to limit its unnecessary use through avoiding false negatives could be valuable. Such a test would exclude patients lacking the disease from receiving surgery and is termed a “rule-out” test. A triage test with this goal in mind should therefore aim to maximize sensitivity. If a patient receives a negative result from such a test, they can be prevented from undergoing costly and invasive surgery with relative certainty of disease absence. Additionally, if symptomatic, the knowledge that they are most likely free of endometriosis can prompt further investigation and aid in uncovering root conditions that may have gone undiagnosed.

In accordance with the Chochrane Review, an ideal test for ruling patients out (i.e. not having endometriosis) should have a sensitivity of at least 95% and a specificity of at least 50%63. The sensitivity is set at 95% assuming a 5% false negative rate is clinically acceptable. The specificity is set at 50% to avoid uncertainty in over 50% of patients receiving a positive result from the diagnostic test. Results for glycodelin suggest that it may be a suitable biomarkers for use as a “rule-out” test. When maximizing both sensitivity and specificity, a cut-off value of 19.8 ng/mL of glycodelin resulted in a sensitivity of 81.6% and a specificity of 69.7% for disease diagnosis. At this cut-off value 81.6% of patient receiving a positive test result would be true positives. Using this, any patient with a serum glycodelin concentration above 19.8 ng/mL could go on to receive confirmation through laparoscopy, while those with a lower glycodelin concentration could be prevented from receiving such surgery. However this cut-off value results in an 18.4% chance that a patient with a negative test result actually has the disease (false negatives) and could thus be prevented from receiving helpful treatment. When sensitivity was maximized to eliminate false negatives, a cut-off value 5.55 ng/mL of glycodelin led to a sensitivity of 100%, resulting in zero false negatives. However this also resulted in a specificity of only 30.4%. The closest to the acceptable level for a triage “rule-out” test achieved by glycodelin came at a cut off value of 9.58 ng/mL. This resulted in a test with a sensitivity of 97.4% (false negative rate of 2.6%) and a specificity of 43.5%. While the specificity is not at the level desired to remove uncertainty in those who receive a positive test result, those who receive a negative result can be quite confident with their diagnosis, thus preventing unnecessary surgical interventions.

The panel of glycodelin combined with ZAG presented in this study is additionally quite close to an acceptable level of accuracy for use as a “rule-out” triage test. In our study population the use of these two biomarkers resulted in a test with a sensitivity of 90% and a specificity of 65%. Again while this does not meet the requirements put forth, the results are certainly promising and should promote further investigation.

While a negative result from a test with high sensitivity is beneficial to avoiding false negatives, a positive result has less diagnostic value, particularly when specificity is low. As opposed to a “rule-out” test, a “rule-in” test maximizes specificity to avoid misdiagnosis. A test with high specificity results in few false positives and so can rule patients “in”. In other words, a positive test result from a test with high specificity would indicate a high likelihood of having the disease. A “rule-in” test can be beneficial for prioritizing which patients go on to receive surgical treatment. A positive result from such a test could also provide rationale for targeted treatment of disease (ie. hormonal contraceptives) without the need for invasive surgical diagnosis. Surgical treatments could then be saved for cases where alternative treatment options fail. This would be particularly beneficial to patient populations at greater risk of surgical complications such as young women, women with medical conditions, or women who are pain-free but have a history of infertility63. An ideal “rule-in” triage test would have a sensitivity of 50% or greater and a specificity of 95% or greater following the same reasoning outlined for a “rule-out” test.

The results obtained in this study for ZAG indicate that it may be suitable for use as a “rule-in” triage test for patients with endometriosis. In this study population, sensitivity and specificity were maximized at a cut-off value of 91.6 pg/mL, which gave results of 46% and 100% respectively. While the sensitivity of this marker for detection of endometriosis is below the 50% threshold, the fact that it can perfectly detect the presence of disease above this cut-off value is quite promising. Additionally, if the cut-off value of ZAG was lowered to 79.9 pg/mL, the sensitivity could be raised slightly to 48% while maintaining a high specificity of 95.7%.

**4.5 Strengths and Limitations**

The strengths of our study include the prospective case-control design, confirmation of endometriosis diagnosis by surgery and pathology, inclusion of a treated group of women with endometriosis, assessment of potential confounders (age, menstrual cycle phase, ethnicity, occupation, and smoking status), and the use of symptomatic controls (women undergoing gynecological surgery with a confirmed absence of disease by a surgeon and pathology). Although the results of the present study are encouraging, there are several important limitations that should be taken into consideration. Specifically, as a tertiary care centre for endometriosis, the majority of our patient population presents with advanced stage disease, resulting in a limited sample size of patients with stage I-II endometriosis. As there is often little rationale to operate on women with stage I-II disease we are restricted to incidental findings of endometriosis in women undergoing laparoscopy for other reasons. Due to the same reasons, a further limitation of the study is the sample size of the control group (n=25). While using only symptomatic controls eliminates potential for misclassification, it also makes it much harder for patient recruitment. Similar to the problems with recruitment of women with stage I-II endometriosis, controls are generally obtained when laparoscopy and histology of women believed to have endometriosis come back negative. Recruitment of symptomatic controls and women with stage I-II disease remains a challenge that may be best addressed through multi-site investigations with an increased sample size. Additionally, a cohort of healthy asymptomatic controls could be added to increase sample size. However this is best avoided if possible to reduce bias. Lastly, as with any study, results pertain only to our study population. As our most promising biomarkers, glycodelin and ZAG, have been rarely studied for their accuracy for endometriosis detection, replication studies are needed to add external validity.

**4.6 Concluding Remarks**

In summary, glycodelin and ZAG were found to be superior to VEGF, IL-6, RANTES, sICAM-1, leptin or SERPINE2 as single noninvasive biomarkers for the diagnosis of endometriosis in our study population. Furthermore, a combination of ZAG and glycodelin led to the most accurate results, showing a sensitivity of 90% and a specificity of 65%. While the current study was unable to meet the desired accuracy for either a “rule-out” (sensitivity≥95%, specificity≥50%) or “rule-in” (sensitivity≥50%, specificity≥95%) triage test, our results are still quite promising and should promote further investigation into the use of these proteins and biomarkers for non-invasive diagnosis of endometriosis.

**4.7 Future Directions**

While promising, the results of the current study did not find a panel of biomarkers as accurate for disease detection as originally hoped. However, prior to the current study, work in our lab measured any analyzed concentrations of an additional five putative biomarkers for endometriosis in an overlapping patient population113. Of these, brain-derived neurotrophic factor (BDNF) was found to be most promising113. When looking at untreated patients with stage I-II disease, ROC curve analysis revealed that at a cut-off value of 1000 pg/mL, BDNF had a sensitivity of 91.7%, and a specificity of 69.4% for disease detection113. As these results are very promising, future work could look at incorporating it, and other promising biomarkers, into the existing panel.

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