Investigating the pathogenesis of Endometriosis through the use of Bioinformatics

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ABSTRACT

Usman Sajjad

BACKGROUND

Endometriosis is a common gynecological disease with unknown pathogenesis. There is a lack of clear understanding towards the pathogenesis and etiology of the disease, and as a result, a deficiency of effective treatment methods. Past theories and approaches have ignored interactions between genes on cellular level and how processes such as the immune system genetically can influence the development of the disease.

HYPOTHESIS

Bioinformatics, an evolving form of computational technology, can be used to systematically and methodically collate available information on gene expression and relevant transcription factors with aim of identifying the key players in the disease process.

METHODS

Genomic/Proteomic information was inputted into software programs such as Ingenuity IPA, GenEvestigator and Opossum. The software was able to produce network maps of biological pathways and meta-analysis plots of genes/proteins differentially expressed during endometriosis. Common regulatory transcription factors were subsequently identified for these up-regulated genes.

Immunohistochemistry (IHC) work was conducted to validate and analyze the expressional pattern of FOXD3 in endometrial samples of 20 patients (n=20). The expression in 10 normal fertile control endometrial samples (n=10) was compared to 10 endometrial samples from women with active peritoneal endometriosis at two different stages of the menstrual cycle (proliferative phase and window of implantation). The same sets of samples were used to

compare any changes in expression of Androgen receptors (AR) and Progesterone receptors (PR), based on findings we obtained from our bioinformatics work.

RESULTS

We found FOXD3 to be the most common transcription involved in endometriosis, controlling 16 out of 38 up-regulated genes. SPP1, PTGS2, IL-8, StAR and CXCR4 were found to be strongly up regulated in patients with endometriosis. The same pattern of up-regulation genes was seen in gastric cancer. AR and PR were seen to interact centrally with other genes/proteins in the Ingenuity IPA network map.

From the IHC work, we found FOXD3 to be expressed in the luminal epithelium and glandular compartments of the normal proliferative endometrium. In patients with endometriosis, there was an increase in expression of FOXD3 in the luminal epithelium and glands of secretory endometrium.

There was no significant difference between the expressional pattern of AR and PR in the endometrium of normal fertile control women and endometriosis patients.

DISCUSSION

We concluded FOXD3 to be a key player in the pathogenesis of endometriosis. The transcription factor was able to control up-regulated genes on a cellular level in various pathological processes, which contribute to the development of endometriosis. We propose future work is needed to extend the investigative measures of FOXD3 in the pathogenesis of endometriosis and elucidate how it can be a therapeutic target for treatment purposes in a clinical setting.

CHAPTER 1

1- INTRODUCTION

Endometriosis is a common gynaecological disease with unknown pathogenesis. The lack of clear understanding towards the aetiology and pathogenesis of the disease has resulted in a deficiency of novel and effective means to treat endometriosis. There is currently a general acceptance that the endometrium that lines the uterine cavity (eutopic) has a significant role to play in the pathogenesis of endometriosis. There were 8279 articles found when I carried out a Pubmed search with the words “endometriosis” and “pathogenesis” on 05/08/11. In most of these articles, the individual researchers discuss and show how one gene or a small group of genes, or gene products are aberrantly expressed in the endometrium of women with endometriosis and discuss how they may cause endometriosis. They may even test how the alteration of such gene(s) in one type of endometrial cells (usually endometrial stromal cells) alters their function in culture. However, this approach ignores the importance of interaction between all genes and gene products within a cell; cell-cell interaction within the endometrium and the effects of external influences such as the immune system on the endometrium to result in the pathological condition of endometriosis.

Bioinformatics was used to systematically and methodically collate a vast amount of the available information on gene expression, gene function, gene products, and cellular function to identify the key players in endometriosis and also to predict suitable targets to treat the condition. Therefore, in my thesis I have reviewed the evidence available on the endometrial cellular aberrations associated with endometriosis and then with the use of bioinformatics I tried to understand how these processes are interconnected and interlinked. Then I discuss how I identified some transcription factors during this process which are likely to play a key role in regulating these functions. Since there have been no previous reports on these factors in human /animal endometrium, I then show how I progressed to test if one of these transcription factors

(FOXD3) is expressed in human normal endometrium and to investigate whetherit is aberrantly expressed in the eutopic endometrium of women with endometriosis.

1.1-What is Endometriosis?

Endometriosis is defined as the presence of endometrial tissue (both epithelial and stromal cells) outside the uterine cavity, usually on the pelvic peritoneum. (1) The tissue can also be found in areas such as the ovaries (known as endometrioma), as well as the rectovaginal septum, and occasionally, implant in the pleura and diaphragm. Symptoms include pelvic pain and subfertility in women. The condition may be the result of biological or anatomical aberrations of the uterus. (1) Endometriosis is an oestrogen dependent chronic inflammatory disease that affects approximately 2 million women in the UK (1).

It has been estimated the disease occurs in roughly 5-10% of women (1) Studies have shown 50-60% of women and teenage girls had pelvic pain, and there are 50% who suffer from infertility. (2) It is not normally seen before the age of 15 in women or after menopause. (3) However, in recent years patients who are under the age of 20 have been identified with endometriosis due to the use of laparoscopy in the diagnosis for patients with symptoms suggestive of the disease. (3)

1.1.1- Epidemiology (Prevalence and Incidence) of Endometriosis

The prevalence of a condition is the total number of cases for a condition i in the population at a given time. The incidence of a condition is the number of new cases over a period of time, being expressed as a rate. (4) Often groups of endometriosis patients are compared for the measures of prevalence and incidence, such as

1. Symptomatic patients with endometriosis undergoing laparoscopy
2. Infertile patients
3. Asymptomatic patients undergoing procedure unrelated

Infertile patients usually possess the highest prevalence rates ranging from 5-45%. (4) Patients admitted for pelvic pain have a prevalence rate of 5-20% (4) Generally the true prevalence of the condition in the general population is unknown as the diagnosis is based on surgical visualisation which has hindered general epidemiology rates. Variations may occur in the estimates of prevalence rates, with there being differences in the severity of symptoms in patients and with some patients being asymptomatic. (4) Additionally, variations in the age at which childbearing occurs in different populations can also cause differences in prevalence rates. The estimated prevalence in the general population is 1.5-6.2% and the age of incidence peaks at 40 years old. (4) Clearer details into the epidemiology of endometriosis have, however, emerged over the past decade due to advances in diagnostic technology. Some studies have estimated the prevalence to be around 4% of asymptomatic endometriosis patients. (112) Cramer et al in 2002 showed that in the UK 1.3 per 1000 females aged 15-44 years old were diagnosed with endometriosis after the use of advancing diagnostic technology. (5)

Another study by Leibson et al in 2004 reported that out of 8229 women diagnosed by the Rochester Epidemiology Project surgical index, the surgical diagnosis of endometriosis was 11.5% of women over the age of 15 years old, and the rate was higher in women aged 45-54 years old. (6) The highest rate of newly diagnosed endometriosis was seen in women between the ages of 25-34 years old. (6)

1.1.2- Effects of endometriosis on society and health care costs

There have been several studies investigating how endometriosis-related symptoms mpact on the physical and mental health of women and on their quality of life. (7) Numerous studies have shown how endometriosis patients had an average of 7.4 hours lost of working time during the week due to the severity of their symptoms. Additionally, 64% of patients had reduced productivity and 60% of patients reported their daily activity had been impaired due to endometriosis. (7) Endometriosis can cause depression in some patients due to the chronic pelvic pain caused, as well as the bowel and bladder symptoms it may result in. (7) Therefore, it is clear the symptoms can

be a substantial burden on a patient's life. (7) As well as incurring a cost of almost £2.8 billion a year (due to days taken off from work), a survey in 2005 showed an 8 year delay in patients being diagnosed from the time they first presented with their symptoms. (7)

1.2- Signs and Symptoms of Endometriosis

Prior to menstruation, the endometrium thickens in response to ovarian hormones. Absence of pregnancy, degeneration of the corpus luteum and a resulting drop in circulating progesterone causes a break down and shedding of the superficial layer of the endometrium. (8) The menstrual flow is known to contain a many inflammatory chemicals (such as prostaglandins, cytokines IL-8, SPP1 (secreted phosphoprotein 1), and others). (8) After menstrual shedding the endometrial tissue undergoes repair, under the influence of hormones. However, in the hypothesis of the pathology of endometriosis, the ectopically situated endometrial tissue and inflammation inducing agents produced during the regeneration process have no way of leaving the body, therefore becoming trapped within the pelvic cavity causing symptoms associated with endometriosis. (8)

The symptoms that arise depend on the site of active endometriosis but generally endometriosis causes a variety of symptoms including severe pelvic pain, painful periods, painful sexual intercourse and infertility. (9) The inflammation involved in endometriosis can stimulate pelvic nerve endings causing pain and may also impair the Fallopian tube function as well affecting the development of oocyte and embryo. (9)

1.2.1-Pelvic Pain

Pelvic pain is a major symptom in patients with endometriosis. (10) The pain may be mild to severe cramping that occurs in the pelvic area, lower back and rectum, progressing to a chronic pain. (10) However, the amount of pain

experienced by women may not correlate with the stage of the condition. (11) Some women may have little pain despite having the condition with extensive scarring. Roth et al in 2011 investigated women suffering from chronic pelvic pain secondary to endometriosis and compared this to women with chronic pelvic pain without the disease. (11) The study found no “psychological disturbance” for women with chronic pelvic pain due to endometriosis. (11) Other symptoms may include dysmenorrhoea, which is a painful, cramp-like pain during menses, getting progressively worse over time. (12) Dysmenorrhoea can include primary and secondary dysmenorrhoea and occurs in 60% of women with endometriosis. (13) Primary dysmenorrhoea usually begins shortly after the first menstrual cycle and will persist until the menopause in the affected women. (13) Therefore, many studies believe primary dysmenorrhoea to be an early sign or manifestation of endometriosis. (13) Other symptoms can also include dyspareunia (painful sexual intercourse) as well as dysuria (urinary frequency and urgency). (14) Gnawing and throbbing pain is also common in patients with endometriosis, and those with a deeper disease compared with superficial, have complained about a greater shooting rectal and pelvic pain. (14)(15) Symptoms reported by some patients have included painful bladder syndrome, irritable bowel syndrome, fibromyalgia, and migraines. (15)

1.2.2- Infertility

Several mechanisms have been proposed to provide a possible explanation into why patients with endometriosis suffer with infertility. (16) Inflammatory changes during endometriosis in the pelvic cavity may affect the peritoneal fluid, which may result in a release of pro-inflammatory factors. The changes to the peritoneal fluid can affect the sperm-oocyte interaction according to some studies. (17) (18) Some published data suggested that the peritoneal fluid from women could cause the immobilisation of sperm due to the action of macrophages and Interleukins 1 and 6. (17) (18) Fertilisation of human oocytes takes place at the distal end of the fallopian tube, and the ampulla of the fallopian tube is exposed to the peritoneal fluid from the pelvic cavity. (17) Studies have also indicated how the increase in inflammatory mediators

during endometriosis such as TNF-Alpha may cause DNA damage to sperm. (19)

Infertility can also be due to dysfunctions in the ovary in patients with endometriosis. With endometriosis extended to the ovaries in some cases, endometriotic cysts can reduce the function of available ovarian tissue. Therefore, there may be a reduced response to ovarian hyperstimulation. (20)

Findings have also shown how the endometrium in the uterus may be altered in patients with endometriosis, possibly affecting fertility in the process. (21) The activation of steroidogenic factor 1 enables prostaglandins to start the expression of aromatase CYP19A1, which leads to *in situ* production of oestrogen. (21) This activity may have an effect on the peristaltic activity of the myometrium and a resistance to progesterone. These changes in the endometrium, may result in impaired implantation of the oocyte, presenting possible issues of infertility. (21) In addition, women with endometriosis have an increased concentration of IgG and IgA, and these antibodies may affect embryo implantation. (18)

Through the study of bioinformatics, we will be studying some of these molecular changes mentioned in women with endometriosis, with the aim of providing a possible explanation. Altered immune response and embryo implantation, as well as oestrogen responsiveness and progesterone resistance will be aspects of endometriosis that we will be looking further into, as well as the genes involved in these processes.

1.3- Diagnosis of endometriosis

Diagnosing endometriosis is a problem in today's primary care setting because patients present with symptoms that are difficult to differentiate from other conditions. Many patients may be misdiagnosed with pelvic inflammatory disease or idiopathic dysmenorrhoea because after menarche, they often present with symptoms associated with menstruation such as a painful period. (22) Therefore, due to the non-specific nature of endometriosis symptoms, problems are created at a clinical level for a general practitioner.

(22)

A large proportion of patients are diagnosed after laparoscopy having had the condition asymptomatically. Symptoms, as mentioned earlier, may develop as early as 15 years old in patients and persist beyond the menopause. (22) Some symptoms are reported more commonly than others. Below is a table of symptoms along with the percentage of women presenting with the symptoms. These findings were based on a study conducted by Louden et al in 1995. (23)

Symptom	Percentage of women with endometriosis presenting with the symptom.
Dysmenorrhoea	40-80
Bloating	42
Lethargy	40
Chronic pelvic pain	20-80
Constipation	29
Lower Back Pain	29
Deep dyspareunia	19-42
Infertility	9

Upon diagnosis, a pelvic examination may be normal but findings may include tenderness in the uterine area, nodularity of the uterosacral ligament and a fixed retroverted uterus. (24) The tenderness often presents difficulty in being able to spot the other features. (24) Additionally, many patients who suffer from fibroids or adenomyosis may also have dysmenorrhoea therefore a diagnosis based on a history of presenting complaint may not be conclusive. (25)

With difficulty diagnosing patients with endometriosis due to the frequent asymptomatic nature and in some cases symptoms resembling other conditions, investigative measures are undertaken clinically.

The gold standard diagnostic test of endometriosis is surgically visualising ectopic lesions of the pelvis at laparoscopy, which is an invasive technique. (24) Endometriosis is usually confirmed through surgery. (26) Nezhat et al in 1994 showed that out of 91 patients with endometriosis who complained of chronic pelvic pain, all had a normal pelvic examination with no tenderness or any positive clinical finding. (26) However, 47% of patients were identified to have active endometriosis after laparoscopy. (26)

Presence of endometrial glands, endometrial stroma, epithelium and hemosiderin macrophages are required before a conclusive diagnosis is made. (27) Lesions found are most likely to be large and have mixed colour in the pelvic cul-de-sac or utero-sacral ligament region. (28) However, studies such as that by Walter et al in 2004 have shown only 55-65% of patients with endometriosis lesions are confirmed through histopathology, and 18% of patients suspected to have endometriosis have no abnormalities identified in the surgically collected biopsies of the ectopic lesions pathologically. (29) Attempts should be made to reach a diagnosis based on taking a history and a clinical examination, rather than immediately proceeding to giving the patient the option of undergoing a laparoscopy.

Transvaginal sonography (TVS) has been used as a non-invasive assessment for patients with pelvic problems. Studies have shown TVS to be a sensitive tool for detecting endometriosis in the ovary. (26) Ovarian endometrioma presents with persistent circular tissue with a demarcation from the ovarian parenchyma. (26)

Moore et al in 2002 reviewed 67 papers on the use of TVS for detecting pelvic endometriosis. It was concluded TVS is a useful diagnostic test for the identification of cystic ovarian endometriosis before surgery, with the prevalence of the condition ranging between 13-40%. (30) Other recent studies have showed how TVS could be used for detecting endometriosis in the uterosacral ligament, the pouch of Douglas and the vagina. (31) TVS is readily available, and is a cost and time effective diagnostic tool, when compared to other procedures such as Magnetic resonance imaging (MRI). (31) TVS can also be used to detect deep infiltrating endometriosis in

patients. (31) Deep endometriosis implants present with regular masses with partings in the centre defined as an “Indian Head dress”. (32)

Recent advances have offered ideas for 3-D sonography, which could increase the accuracy of the assessment of deep endometriosis in diagnosis. A study by Bazot et al in 2003 suggested transvaginal ultrasound could also diagnose colorectal endometriosis, showing if the muscularis propria has been infiltrated in a patient. (32)

Serum markers have also been studied in relation to detecting endometriosis in various past studies. May et al in 2010 investigated the availability of peripheral biomarkers in endometriosis. The study highlighted the delay in making a diagnosis, and the advantages that the availability of biomarkers which may help a clinician diagnose and treat endometriosis in patients would bring. (33) Any marker found could avoid unnecessary diagnostic procedures and allow for cost-effective treatment plans. Therefore further research is needed before one biomarker can be recommended for clinical use. (33) We plan to build on past work and investigate potential biomarkers and transcription factors, with the aim of discovering whether they could be used for diagnosis and treatment in the future.

1.3.1- Clinical Staging of Endometriosis

Endometriosis can be staged I–IV during surgery according to the American Society for Reproduction. (34) The staging system assesses adhesions or lesions in the pelvic area and stages the level of the disease. (34)

Stage I (Minimal) - The findings are usually only superficial lesions and very few adhesions. This is peritoneal endometriosis.
Stage II (Mild) – There is superficial but some deep lesions are found in the cul-de-sac.
Stage III (Moderate) - Superficial, adhesions and deep lesions are present as well as endometriomas.

Stage IV (Severe) – All the above are present, as well adhesions and large endometriomas.

The staging system does not show the level of pain and infertility. Patients may have stage 1 endometriosis whilst experiencing severe symptoms such as pelvic pain and infertility.

1.4 Treatment of Endometriosis

1.4.1- Medical Treatment

The aim of medical treatment in patients with endometriosis is to halt the growth of endometriosis lesions. The most common medical treatment is the use of gonadotropin-releasing hormone (GnRH) agonists and oral contraceptives. (35) This treatment is carried out in patients whom have had a confirmation diagnosis and have had surgical excision, and require long-term management. (35) Oral contraceptives are taken indefinitely, being effective in treating dysmenorrhea. They can help relieve milder symptoms and can be taken over long periods of time.

A study showed how in 57 patients, after 6 months, oral contraceptives reduced the severity of dysmenorrhea compared to GnRH agonists. (36) Pathologically, oral contraceptives cause atrophy of the endometrial tissue; whilst GnRH agonists cause a down-regulation of the pituary-ovary axis so there is a state hypoestrogenism (reduced oestrogen production). (36) GnRH agonists are taken as a nasal spray, implant or an injection, and are taken continuously over two weeks. Side effects include hot flushes and vaginal dryness. (36)

Androgens can induce atrophy of the endometrium, and danazaol (synthetic derivative of 17α-ethinyltestosterone) acts to increase the concentration of circulating testosterone, which is able to inhibit luteinizing hormone (LH) surges and estrogen production from the ovary. (36) An artificial menopause is induced, contributing to the inhibition of ectopic and eutopic endometrial

growth. (36) The fourth hormone taken as part of the medical treatment is medroxyprosterone acetate, which is a type of progestogen. The hormone behaves like progesterone, preventing ovulation, so the endometrial tissue shrinks. (36)

Non-steroidal anti-inflammatories (NSAIDs), such as ibuprofen may also be used to prevent inflammation (pain, tenderness and swelling) from endometriosis, relieving any discomfort and pain. (36)

Hormone production, as can be seen, limits the production of oestrogen in the body; therefore endometrial tissue size is reduced so symptom severity is lessened. However, medical treatment does not have any effect on the adhesion of ectopic endometrial tissue and can also decrease fertility.

1.4.2- Surgical Treatment

Hormone therapy is not effective in combating the effects of endometriosis on other organs and therefore laparoscopic surgery (mentioned earlier in chapter) may be needed to remove endometrial tissue growth and scar tissue. (37) Surgical therapy can be performed alongside diagnostic laparoscopy. In the advanced stages of endometriosis (Stage 3 or 4 according to the American Fertility score), laparoscopic surgery is used to remove visible endometrial implants and divide scar tissue. (37) This is because lesions larger than 3cm respond poorly to hormonal treatment so surgical intervention is needed. The laparoscope is inserted into the pelvic cavity through a small cut near the naval, and a light source allows the gynaecologist to view the organs in the pelvic region and any ectopic endometrial tissue implants present. (37) A laparatomy is a procedure in which a larger cut (10-15cm) is incised into the abdomen if the severe endometriosis cannot be treated by laparoscopy. If minimal to moderate endometriosis is found, a diagnostic laparoscopy can be combined with an operative laparoscopy. (37)

The removal of the uterus (hysterectomy) or removal of the ovary

(oophorectomy) may be an option depending on the severity of the condition as well as the age of the patient. (37)

A Cochrane review conducted by Furness et al in 2011 reviewed the “Pre and Post Operative medical therapy for endometriosis surgery”. (38) There was no evidence of benefit associated with post surgical medical therapy and there was not enough evidence to determine whether there was a benefit for pre-surgical medical treatment. (38)

With no treatment suitable for all patients and not effective in some cases, more understanding is needed regarding the pathogenesis of the condition. Even though there is not a full understanding of the aetiology and pathogenesis of the condition, there are available theories and explanations that have been put forward by studies in the past few decades.

1.5- Aetiology and Pathogenesis of Endometriosis

The most commonly held theory on its aetiology is that retrograde menstruation and trans-tubal migration of viable endometrial fragments occurs into the pelvic cavity where they attach to and invade the peritoneal mesothelium establishing ectopic growth of endometrial tissue (39). Given that most women reflux menstrual effluent into the peritoneal cavity during menstruation, and endometriosis only develops in 10-15% of women, it is unclear why some women develop endometriosis whilst others do not. (39) Endometriosis is diagnosed in 20% of women who have undergone laparoscopy for pelvic pain or infertility. (39) The pregnancy rate in women with endometriosis is about half that of women with tubal factor infertility and decreases as the severity of endometriosis increases (39).

There are three theories of the pathogenesis of endometriosis. The Sampson’s Theory was originally devised in 1920 and states that during each menstruation; some of the blood goes in the opposite direction and flows out of the fallopian tubes, proposing endometrial fragments are spread by retrograde menstruation through the fallopian tubes into the peritoneal cavity. (40) The theory states that as the blood is flowing out towards the fallopian

tubes, it carries cells from the lining of the uterus. These cells eventually implant onto the surface of the pelvis and grow to form ectopic endometrial implants, accounting to the development of endometriosis. (40)

The pattern of endometriosis supports this theory and endometrial fragments gravitationally move to the area of the pelvis including the ovary, utero-sacral ligaments and the fallopian tubes (41). The endometrial fragments will implant on the peritoneal surface and causes an inflammatory response, followed by angiogenesis, adhesion, fibrosis and scarring. (41) Endometriosis can also be inherited polygenically, with evidence of linkage to chromsomes 7 and 10. (41)

The second theory of pathogenesis is the coelomic-metaplasia hypothesis and this suggests that there is a differentiation of mesoepithelial cells into endometrial like tissue. (42)

A third theory suggests that the menstrual tissue from the endometrial cavity can spread to the rest of the reproductive system via lymphatic vessels and veins, and that circulating blood cells originating from bone marrow can differentiate into endometriotic tissue at various sites. (41) This is supported by some patients possessing deep rectovaginal endometriosis showing that the disease can spread via the lymphatic system, as well as cells being present in other parts of the body. (43)

Endometriosis produces inflammatory mediators, which cause pain and inflammation resulting in scarring of surrounding tissue. (41)

Other possible environmental factors can cause endometriosis. There have been studies to show Dioxin exposure to be a likely cause of endometriosis, with Rier et al study in 1993 showing 80% of monkeys had developed endometriosis from receiving a dose of dioxin. (44) The study also outlined how other pesticides may produce a hormone imbalance, possibly contributing to endometriosis. (44)

Studies have also shown that the risk of endometriosis may be reduced in smokers. (45) Somigilana et al in 2011 showed that smoking causes a decrease in estrogen with increased bleeding and shorter luteal phase. (45) With endometriosis being an estrogen related disease, this may be a plausible link.

With commonly existing theories into the etiology of the condition present, various biological processes that are affected by endometriosis provide us with a possible explanation into the pathogenesis of the condition. We looked to use the genetic molecular information found from different pathological processes involved in endometriosis (obtained from a PubMed literature search) and use this in a bioinformatics approach. This was able to direct us later in the research project to gain a better understanding of the pathogenesis of the condition and provided information on key players, which may be targeted for future treatment purposes.

1.5.1 Molecular aspects and physiology of the normal endometrium

The endometrium is the innermost glandular layer and functions as the lining for the uterus. During the menstrual cycle, the endometrium will grow and thicken to form a glandular like tissue layer, presenting an optimum environment for the blactocyst to implant upon it arriving to the uterus. (16) The endometrial glands and blood vessels in the endometrium increase in size and number during pregnancy. The molecular mechanisms of implantation in the normal physiology are only partially understood. There is a cascade-like interaction between the embroyonic trophoblast cells, epithelial cells, decidual cells, the extra-cellular matrix (ECM) of the maternal endometrium and the cells responsible for immune reactions in the normal endometrium.

The blastocyst secretes molecules such as EPF (early pregnancy factor) and HCG (human chorionic gonadotrophin), which affect the activity of the endometrium and ovaries. Cadherins are cell adhesion molecules which play

a role in anchoring the blastocyst in its route towards the endometrium in the pre-implantation stage. (16) The embryo will produce interleukin 1 (IL-1) which is important in orientating the embryo towards the endometrium. The embryo also produces Platelet-activating factor (PAF). In the pre-implantation phase, the glycocalyx (surface proteins) and the decrease in the electrostatic repulsion between the blastocyst and the endometrium, allows for the facilitation of implantation in the normal endometrium. (17) The secretion of implantation factors, Interleukin 1 (IL-1), the inhibition factor for leukocytes (LIF), colony-stimulating factor (CSF), as well as the epithelial growth factor (EGF) and its receptors (EGF-R), allow for adhesion of the blastocyst onto the uterine epithelium.

During implantation in the normal endometrium, IL-1 binds on the receptors at the surface of the epithelial cells. LIF is synthesized on the 18th day of the menstrual cycle and the receptors are expressed on the blastocyst.

Upon implantation, the basal membrane of the trophoblast is destroyed and grows into the decidua of the uterine tissue. The cells of the trophoblast secrete enzymes such as MMP (Matrix Metalloproteinases) and plasminogen-activators. The trophoblastic cells express integrins on the cell membrane and these are able to interact with the uterine muscoca. Subsequently, the trophoblast is able to grow into the endometrium and the extra-cellular matrix is able to decompose under the influence of endometrial factors that are secreted by epithelial cells, fibroblasts, macrophages and leukocytes. These factors, which may be aberrantly up or down regulated in patients with endometriosis, can cause an autocrine and paracrine effect on the normal endometrium.

In patients with endometriosis, these outlined molecular activities observed in normal endometrium may be notably different.

1.5.2- Molecular aspects and physiology in endometriosis

1.5.2.1- Increased cell adherence

Endometrial cells in women with endometriosis have been shown to demonstrate increased adherence to peritoneal cells. Griffiths et al in 2010 shown how there is an increased expression of CD44, which is an important cell adhesion protein. (46)

According to many other studies (Morin 1999), increased cell adherence signaling has been demonstrated in endometriosis. (47) (48) The study shows that there is an increase expression of cell adherence molecules and enzymes such as Beta catenin, E-Cadherin and P-Cadherin. (47, 48)

Increased cell adherence possibly plays a major role in the Sampson's theory of retrograde menstruation with endometrial fragments being found outside the uterine cavity, and the involvement of molecules such as CD44 may play an important role in that. We searched for genes and proteins involved in adherence through our literature search of "retrograde menstruation".

1.5.2.2-Metastasis

Since endometrial tissue survives in unusual locations (outside of the normal site of uterine cavity) it can be regarded as a metastatic disease. However, endometriosis is not fatal or malignant. Described as a benign metastatic disease, we reviewed studies to discover any genes that are involved in metastatic changes within patients with endometriosis. Metastasis is the spread of a disease from one organ to another or to an adjacent part of another organ. (49) Endometriosis exhibits features of adherence, invasion and metastases. (49) Endometriosis can be characterized in patients histologically by endometrial glands with cytological atypia, and has been observed in 12-35% of ovarian endometriosis cases. (50) There have been seen to be many similarities in the metastases involved in endometrial cancer and endometriosis. Invasive cancer is distinguished from non-invasive cancer through the ability of cells to invade through the basement membrane. (50) Endometrial carcinomas have been seen to secrete proteases and expression of these proteases such as matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) can be linked to grading and stages of the

cancer. (50) Similarly in endometriosis, MMP activity has been shown to be up-regulated in endometriosis patients. (50)

Specifically, MMP-1 and MMP-2 are increased within the eutopic endometrium of patients with endometriosis. (51) Local MMP factors such as MMP-1, MMP-2 and MMP-9 can degrade the extracellular matrix of the endometrium and cause vascular rebuilding, resulting in a migration of endothelial cells. (52)

Studies such as that of Ning et al in 2006 showed that there is higher expression of MMP 1, 2, and 9 in the eutopic endometrium of women with endometriosis compared to normal patients.(53) The expression of MMPs is controlled by cytokines and steroid hormones in women. MMP-1 is detected during the early stages of the menstrual cycle, whereas MMP-2 is expressed during the entire menstrual cycle. (54)

Therefore we can predict MMP molecules have a role in the pathogenesis of endometriotic lesions and metastatic activity.

1.5.2.3- Proliferative Activity

In endometriosis, patients have been seen to possess increased levels of endothelial growth factor molecules. Studies such as that by Burlev et al in 2010 indicated how patients have elevated levels of vascular endothelial growth factor A (VEGF-A), VEGF-1 and VEGF-2. Therefore there is increased angiogenesis activity in patients. (55) Other studies from the same author mentioned above investigated apoptosis and proliferative activity in the glandular epithelium of the endometrium in patients with endometriosis. (56) The study investigated whether the proliferative activity of the glandular epithelium in the proliferative phase of endometriosis was higher than that in the secretory phase. (56)

Studies have shown the increase in expression of specific molecules have had an effect on the proliferative activity of the endometrium in women with endometriosis compared to those who do not have it. Park et al in 2009 examined 631 infertile women, including 197 without endometriosis and 434 with the disease. (57) It was found there was an increased expression of Ki-

67 in assay results from patients with endometriosis. (57) Therefore, there was seen to be an increased proliferative activity in patients. (57)

As mentioned earlier, it is known physiologically the endometrium thickens during the proliferative phase of the menstrual cycle in response to oestrogen. During ovulation, the endometrium will provide a host for the attachment for the early embryo until the placenta develops into the early stage of pregnancy. Studies such as that of Jason et al in 2008 investigated how in the infertile population, there may be a defect in the proliferative phase of endometrium under the influence of Follicle Stimulating Hormone (FSH). (58) In the proliferative phase, FSH is secreted by the anterior pituitary gland. (58) The secretion rises in the last few days of a women's menstrual cycle and this rise will cause the production of ovarian follices. FSH causes proliferation of granulosa cells in the developing follices and an increase in the expression of luteinizing hormone (LH) in the granulosa cells. (58) The granulosa cells will begin to secrete oestrogen.

Jason et al's study in 2008 investigated how patients with a diagnosis of PCOS (Polycystic Ovarian Syndrome) or endometriosis achieved a lower peak endometrial thickness than control subjects in the study. (58) However, Zhang et al in 2010 discovered that 17β-estradiol promotes cell proliferation in the endometrium in endometriosis by activating the PI3K/AKt pathway via an NFκB/PTEN-dependent pathway. (59)

1.5.2.4- Apoptosis

Apoptosis is a biological process involving programmed cell death within organisms. (60) This process eventually leads to cell changes and death. (87) The changes include DNA fragmentation and cell shrinkage, as apoptotic bodies are produced. (60) (61) Apoptosis is important in removing senescent endometrial cells from the functional layer of the endometrium. (62) Senescence is the morphological change in a cell or organism after it ages.

Apoptotic bodies are able to remove cell contents after being engulfed by these bodies. (61) Apoptosis, therefore, is important in removing senescent

cells. (62) The process is important in the normal endometrial function during the menstrual cycle in maintaining homeostasis. (61) Studies have shown that there is decreased apoptotic activity in the endometrium of women with endometriosis compared to patients who do not have the condition. (61) Therefore, there is a decreased removal of unwanted cells, and there is an increase of cells in the endometrium.

As mentioned before, the higher expression of Bcl-2 has an effect on the decreased apoptotic activity in patients with endometriosis. With endometrial cells from patients having a greater ability to implant and survive elsewhere ectopically due to increased proliferation, the lower apoptosis activity allows for these cells to survive longer. Studies, such as that by Meresman et al in 2000, investigated that there was a decrease in expression of apoptotic bodies such as BAX and an increase in expression of Bcl-2, which is an anti-apoptotic factors. (63) BCL-2, a molecule seen to be elevated in patients with endometriosis, encodes an outer mitochondrial membrane protein suppressing apoptosis in numerous cells. (63) Bcl-2 is seen to work in a feedback system with caspase molecules, by inhibiting caspases. (89) Other members of the BCL group are seen as pro-apoptotic (promoting apoptosis), whilst other's such as BCL-2 and Bcl-xl mentioned to be elevated in patients with endometriosis, are anti-apoptotic. (63)

Therefore, the increase in proliferation and decrease in apoptosis with more endometrial cells being present to be refluxed supports Sampson's theory mentioned earlier. Genes related to apoptosis such as GADD454 and GADD45B have been found to be down regulated in the endometrial tissue according to a study conducted by Eyster et al in 2007. (64)

With studies showing a promotion of cell proliferation of the endometrium in patients and a decrease in apoptosis, we decided to look into genes and molecules that were involved with this. From there, we used bioinformatics technology to investigate how these genes may be linked to other genes involved in pathological processes in patients with endometriosis. This is important because the genes mentioned in previous studies eg BCL-2, BAX

etc play an important role in proliferation and apoptosis, and could lend support to some of the suggested theories of pathogenesis such as retrograde menstruation. With studies showing clear differences in endometrial cell activity between women with endometriosis compared to normal patients, conducting Pubmed searches to discover genes that are responsible was important. These searches to find more genes and molecules helped provide a greater insight into the pathogenesis of the condition.

1.5.2.5- Oestrogen responsiveness

I conducted searches on studies that have investigated oestrogen responsiveness and its involvement in endometriosis. It is known that granulosa cells secrete oestrogen and the increased levels of oestrogen (during the proliferative phase of the cycle) stimulate the production of gonadotropin-releasing hormone (GnRh), and this also increases the production of LH. (61) The increased production in LH stimulates the production of androgens in thecal cells of the endometrium and stimulates proliferation of endometrium, therefore the rising levels in the blood cause growth of the endometrium and the myometrium in the uterus. (61) As well as being secreted by the ovaries, peripheral formation from the increasing concentration of circulating estrone sulphate can also increase the amount of oestrogen. (65) Studies have shown that there is abnormal expression of oestrogen-metabolizing enzymes resulting in high estrogen-2 (E2) biosynthesis leading to increased proliferative activity of the endometrium in patients with endometriosis. (65) The oestrogen formed (E2) will bind to both oestrogen receptors (ERα and ERβ). (66)

In patients with endometriosis, studies have shown that there is higher expression of ERα compared to that of ERβ in the eutopic endometrium of endometriosis patients. (94) The levels of both of these receptors are higher in the proliferative than the secretory phase. (94) The levels of ERβ are higher

in the ectopic endometrium due to abnormal DNA methylation of the promoter gene for the ESR2 receptor. (67)

Rizner at al in 2009 studied oestrogen metabolism and the effect of endometriosis. (68) It is known that endometriosis will progress in an oestrogen-dependent manner, according to Kitawaki et al in 2003 who showed that an increase of oestrogen can cause a relapse of endometriotic lesions. (69) Other studies such as that by Takahashi et al found that patients with endometriosis were found to have higher levels of estradiol (E2) in comparison to healthy women.

The rise in oestrogen also causes endometrial cells to produce progesterone receptors, helping the endometrium to respond to rising levels of progesterone during the late proliferative phase. (69)

Therefore, we were able to find any possible genes or molecules related to oestrogen responsiveness in patients with endometriosis, with aim of helping us achieve a greater understanding of the condition on a molecular and genetic basis.

1.5.2.6- P450 Enzymes

P450 is a cytochrome and is part of a large group of enzymes. They function by catalyzing the oxidation of substances such as lipids and steroid hormones. (70) P450 enzymes use large and small substrates in enzyme reactions, and are at times part of electron transfer chains. (70) In the same paper mentioned earlier by Kitawaki et al, the aromatase cytochrome P-450 was studied and its possible detection as a diagnostic test for endometriosis. (69) It was found that the expression of aromatase cytochrome P-450 in biopsy specimens of eutopic endometrium in patients with endometriosis is distinguishable from patients who do not have the condition, in which P-450 was not expressed. (69) Therefore it is also vital we investigate microarray studies that have studied P-450 and patients with endometriosis.

Other papers have shown how there is increased expression of the mRNA of the aromatase enzyme in the ectopic endometrium of patients with endometriosis. Studies by Aghajanova et al in 2009 and Smuc et al in 2007, showed increased expression of P450 enzymes in ovarian, deep and peritoneal endometriosis. (71) However, according to Heilier et al in 2006, higher levels of aromatase p450 enzymes were found in ovarian endometriosis, compared to that of peritoneal and deep endometriotic nodules. (72) In ovarian endometriosis, over-expression of the P450 aromatase enzymes assists the catalyzing of androstenedione to estrone (estrogen), as well as testosterone to estrogen-2 (E2). (72) Therefore, this indicates the P450 enzyme is important in the synthesis of estrogen and we used this as a search category to find particular genes or molecules that were involved in the pathways of P450 and estrogen synthesis.

1.5.2.7- Prostaglandins

Prostaglandins (PGE2) are lipids that are important in producing an autocrine or paracrine response through binding to a G-protein coupled receptor to activate intracellular signaling. (73) There is great importance in ovulation, implantation and menstruation. Prostaglandins are also important in augmenting contractions within the uterus, through increasing intracellular calcium ions. They also interact with involvement of oestrogen and fibroblast growth factors (FGF)-9 production. (73) Subsequently, angiogenesis is promoted as endothelial cells are recruited to form new vasculature so in every menses, prostaglandins contribute to tissue repair, growth and differentiation within the uterus. (73)

Prostaglandins are produced through the conversion of arachidonic acid to prostaglandin H2 and this is regulated by cyclooxygenase (COX), which has two isoforms, COX-1 and COX2. In the peritoneal macrophages of women with endometriosis, COX-2 is expressed in greater quantities, whereas in severe stage endometriosis COX-1 is expressed. (74) Therefore the expression of COX enzyme in the peritoneal macrophages is linked to the

concentrations of PGE2 in the peritoneal fluid within the womb of the women and also the severity of endometriosis. (74).

In many cases, there has been a higher expression of COX-2 in ectopic endometriotic lesions, with increased production of prostaglandin E2 from primary cultured stromal cells in the ectopic endometriotic lesions. (75). In contrast, it is also thought that pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-alpha) and interleukin- 1beta (IL-1Beta) can cause over-expression of COX-2 in women with endometriosis. (76) The feedback loops involving pro-inflammatory cytokines such as IL-1beta and TNF- alpha in peritoneal macrophages and prostaglandin E2 can cause the over expression of COX-2 in peritoneal macrophages and ectopic endometriotic stromal cells in women with endometriosis. (76)

Enzymes such as steroidogenic acute regulatory protein (StAR) and P450 aromatase (as mentioned earlier) control the steps in the production of oestrogen. Prostaglandin E2 plays a role in inducing StAR and aromatase in the endometriotic stem cells, which results in the spontaneous production of oestrogen. (77). Prostaglandin E2, upon the rise in concentration of peritoneal fluid, binds to the G-protein coupled plasma membrane receptors. (77) There are 4 different plasma membrane receptors, and binding of PGE2 to Receptor 2 and 4 will activate adenylyl cyclase and protein kinase A (PKA), causing a Ca2+ influx and myometrial contractility. (78).

Therefore, it is important we conducted searches for other important genes and molecules through a bioinformatics approach with the aim of linking the processes mentioned above to what occurs in endometriosis.

1.5.2.8- Calcium signalling and uterine contractility

I also investigated genes and proteins that are important in the calcium-signalling process within the endometrium and myometrium of women with endometriosis. With calcium signalling resulting in contractions of the uterus,

there is a possibility according to a study by Bulletti et al that uterine contractions may increase the intensity of symptoms in patients with endometriosis. (79) In addition, aberrant myometrial contractility may aid retrograde menstruation. Therefore proteins or genes found that express certain ion channels or transmembrane receptors should be investigated.

Myometrial contractility requires phasic influx and efflux of calcium ions (Ca^{2+}) across the membrane. Ca^{2+} efflux occurs via Ca-ATPase and Na^+/Ca^{2+} exchanger. (80) The transmembrane sodium ions (Na^+) and Ca^{2+} work together with the membrane voltage and dictate the direction of Ca^{2+} fluxes. (80) Calcium activated potassium K^+ channels (Kca) are also sensitive to the flow of Ca^{2+} ions and the membrane voltage. (80) These channels are important in contributing to the resting membrane potential of the smooth muscle in the myometrium due to the large conductance and high Ca^{2+} sensitivity. (81) Studies have shown that blocking Kca channels may influence the contraction of the myometrium. (81) Ca^{2+} activated chloride channels have also been reported in the myometrium and produce depolarisations and the main source of Ca^{2+} for contraction is entry through voltage-sensitive L-type Ca channels. The entry of Ca^{2+} causes further depolarization of the membrane potential. Entry of Na^+ via an agonist-stimulated non-specific cation channels will also depolarize the membrane. (81)

In the myometrium, contractions can occur through inositol triphosphate (IP3) receptor mediated release. This occurs via an agonist such as prostaglandin or oxytocin binding to receptors on the surface membrane, subsequently activating phospholypase C via GTP-binding proteins and hydrolysing the phosphatidylinositol 4,5- bisphosphate (PIP2) in the cytosol. This results in the production of IP3 and a release of Ca^{2+} from the sarcoplasmic reticulum (SR). (82)

In addition to the plasma membrane L-type Ca^{2+} channels, there is also an internal Ca^{2+} store the sarcoplasmic reticulum (SR). Its Ca^{2+} may be released into the cytoplasm to augment contractions. (81) The release of Ca^{2+} is mediated by an increase of IP3, which subsequently binds to the receptors on the SR membrane. (82) SERCA, which is a P-Family Ca-

ATPase channel, allows the uptake of calcium ions from the cytoplasm into the lumen of the SR via the use of ATP against the Ca gradient. (82) SERCA2b is the most abundant of the three isoforms within the smooth muscle of the uterus. (81,82) According to a study by S.Wray/A Slunygol, it was suggested that there is an increased rate of calcium ion accumulation in labouring myometrium as both SERCA2b and SERCA2a were found to be in increased expression. Earlier work by Taggart and Wray (1998) also suggested that inhibiting SERCA might result in an increase in calcium ion movement, therefore an increase force of contraction, as there is an inhibition of entry of calcium ions into the sarcoplasmic reticulum. (82)

1.5.2.9- Altered Immune Response

We also considered the role of the immune system and immune related inflammatory mediators that may be raised in patients with endometriosis. Genes or molecules that may be involved in an altered immune response in patients with endometriosis may help us with understanding the pathology. Gilmore et al in 1992 investigated how lymphocytic activity may be different in women with endometriosis compared to women who do not have the condition. (83) The study found how the proliferation of lymphocytes was lower in women with endometriosis compared to controls. (83)

Studies have shown how the peritoneal fluid in women with endometriosis usually contains an increase in a number of cytokines. A study by Piva et al in 2003, showed how an increase in inflammation causes an increase in IL-1, IL-6, IL-8 and tumor necrosis factor alpha. (84) As well in the proliferative and secretory phase of the menstrual cycle, studies such as that by Khan et al in 2004 have shown there to be an increase in the number of macrophages. (84)

Therefore, the suspected rise in inflammatory mediators may be responsible for the other pathways such as proliferation and those mentioned earlier in the report. Studying the genes and proteins involved in an altered immune response in patients with endometriosis through a bioinformatics approach will help us achieve a greater understanding into how the symptoms may arise.

1.5.2.10- Embryo Implantation

We also looked at genes and molecules involved in impaired embryo implantation in women with endometriosis. With little known about infertility , we hoped a bioinformatics approach would help us create a better understanding. Studies, such as that of Burney et al in 2007, investigated how there was a down regulation of factors, such as MUC-1 and osteopontin, involved in embryo attachment in patients with endometriosis compared to normal controls. (85) MUC-1 is a glycosylated phosphoprotein that is important in adhesion during implantation. (85) Another study by Arici et al in 1996 looked at the effect of endometriosis on implantation. (86) The study found that implantation rate (gestational sac per transferred embryo) was lower in endometriosis compared to a group with unexplained infertility. (86)

Cho et al in 2009 studied the osteopontin mRNA expression in the eutopic endometrium of patients with endometriosis. (87) Osteopontin (OPN) has important functions in immune responses such as apoptosis and bone remodelling, and has been shown to be involved in embryo implantation in past studies. (88) Cho et al's study investigated 79 patients with endometriosis and 43 without the condition. (88) In contrast to what was found by Burney in 2007, Cho et al found that Osteopontin expression was higher in women with endometriosis compared to controls. (88) Unlike Burney's study indicating the down regulation has an effect on implantation, Cho suggested the increased expression will have an effect on the pathogenesis of the condition and should be used as a diagnostic marker. (88)

The studies outlined the various pathological processes showing a variety of genes aberrantly expressed in patients with endometriosis, however there is no clear or coherent explanation into why and how endometriosis occurs. Theories such as retrograde menstruation, and evidence of linkage to

chromosomes 7 and 10 have emerged, however computational technology is needed to collate all the data found in different pathological pathways to create mapping systems and imaging. Therefore Bioinformatics has allowed us to collate the genetic information through various high-technology programs, and provide a greater understanding into pathways and find particular genes/molecules in these pathway that are affected. Bioinformatics provides an opportunity to identify the key players in the pathogenesis of the condition, with aim of targeting these particular genes/proteins in future therapeutic purposes.

1.6-What is Bioinformatics?

Bioinformatics is a field of scientific research, which merges with computer technology, carrying the aim of discovering new insights into a biological process or a medical condition. (89) At the start of the “genomic revolution”, the importance of bioinformatics grew because a database was needed to store biological information such as amino acid and nucleotide sequences. (89) It became clear recently that all this information can be combined so a full comprehensive picture can be formed into how particular biological pathways and cellular activities function and how they may be altered in certain disease states, such as endometriosis. Analysis is completed through the development of mathematical formulas and statistical information allowing for a study of the relationship of large sets of biological and molecular data through programs such as Ingenuity IPA. (90)

Mishra et al in 2010 investigated lung cancer through a bioinformatics approach, using the Ingenuity IPA Software program. (90) The study involved using Ingenuity to investigate the RAS subfamily, which includes a family of proteins that cause over-expression of cancer causing genes (M-ras) resulting in the formation of a tumor in the lung. (90) Similarly, using information on testicular proteins from a protein database, Fu-Jun et al in 2011 performed a bioinformatics and Pathway analysis integrating the data into a comprehensive functional network (91). The study used the clustering of particular testicular proteins, and compared the organization of these clusters

to positional clustering of genes on the chromosomes. (91) The biological interpretation of testicular functions in a network context provided a greater understanding of the physiology. (91)

Bioinformatics has also been used in studies to calculate the substitution rate in genes. (92) The study by Mank et al in 2009 aimed to carry out analysis of sex-biased genes and the study used a bioinformatics approach following the input of micro-array data, which compared the male and female gene expression in different chicken embryo tissues. (92) The study found that from the 15982 significantly expressed coding regions assigned to autosomes of the Z Chromosome, approximately 18% were sex biased in any one tissue. (92)

Microarray technology used in this and many other studies opens the possibility to study expressional patterns, increasing understanding towards genomic mechanisms. (92, 93) The principal behind microarray analysis is initially the hybridizing of two DNA strands so nucleic acid sequences will pair with hydrogen bonds between nucleotide base pairs. In these strands, more complementary base pairs in a nucleotide sequence means there is a tighter bonding (non covalent) between the two strands. (94) Fluorescently labeled target sequences bind to a probe sequence, and expression analysis applications such as gene expression profiling, single nucleotide polymorphism (SNP) detection and geneID are used to detect DNA/RNA that may or may not be translated into a protein. (93)

Eyster et al in 2005 investigated the DNA microarray analysis of gene expression markers in endometriosis. (94) The study showed how the expression of 8 genes from a total of 4,000 or so genes on the DNA microarray was increased in endometriosis implants in patients compared to the uterine endometrium of normal women. (94) It also showed how DNA microarray analysis is an effective tool for identifying differentially expressed genes between uterine and ectopic endometrium. (94)

In this research project, we planned to review the data from various other studies that have produced targets or specific genes, which are expressed or have been identified as being involved in the pathology and aetiology of endometriosis.

With little known into the pathogenesis of endometriosis, bioinformatics was used to provide a plausible genetic explanation and understanding into the disease. Our aim was to provide a breakthrough into specific genes, proteins and transcription factors that are involved in the various biological processes that occur in patients with endometriosis and show how they may be interlinked. Most investigators feel that endometriosis is inherited in a polygenic/multi-factorial mode however the genetics into the condition is still complex and largely unknown. (95)

We used tools such as Ingenuity, Genevestigator, Opossum and UniProt as part of our bioinformatics approach. These programs are explained in Chapter 2: Methodology.

1.7- Transcription and Transcription Factors

As well as looking at genes that may be responsible for the functioning of endometriosis and the processes involved within the condition, we will also be investigating factors that control the expression of these particular genes that largely depends on the transcription and then translation. (96) Regulation of transcription is important in gene expression, and mRNA (messenger RNA) is often used as an indication of a gene activation with the assumption that the mRNA will be used for a translation of encoded protein in the comparably short time window (97).

We investigated the transcription factors that control the expression of key genes involved in endometriosis. Transcription factors are sequence-specific DNA-binding factor proteins that are able to bind to DNA sequence during transcription, and therefore regulate the flow of genetic information from DNA to mRNA. (98) Transcription factors function through promoting or inhibiting the recruitment of RNA polymerase to specific genes. (98)(99) Transcription

factors possess a DNA binding domain, which is essential in specific sequences of DNA binding to them for regulation. (99)

According to Babu et al, there are 2600 proteins containing DNA-binding domains in the human genome and they function as transcription factors. (100) Many genes possess several binding sites for unique transcription factors, and on some occasions, the expression of these genes require the action of many different transcription factors working at once. (99)

Transcription factors have many functions physiologically. Many transcription factors, such as those involved in ovarian or endometrial cancer are known as Oncogenes and Tumour suppressors. They regulate the cell cycle of cancer cells. (100) An example of an Oncogene is Myc, which is important in cell growth and apoptosis. (101)

Transcription factors are also important in development of cells within organs, and function by turning on or off the transcription of certain genes, allowing changes in cellular activities to occur for differentiation within the tissues. (101) Lemons et al in 2005 studied how the HOX transcription factor family is important in the anatomical development and in cascade systems switching on the transcription of other genes.(102) Other transcription factors that are important in development are those such as factors encoding the Sex-Determining Region (SRY) gene. (103)

In relation to the female reproductive system and its physiology, there are many transcription factors that are involved in signalling cascades regulating genes involved in endometriosis. If a signal within a cascade requires a gene to be up or down regulated in a cell, transcription factors will usually be downstream in the cascade. (103) As mentioned earlier, endometriosis is an oestrogen-dependent condition, and the signalling requires oestrogen-receptor transcription factors. (104) After being secreted by the ovary or placenta, oestrogen binds to the receptor in the cytoplasm and then will bind to the DNA-binding site. (104) It is likely, therefore, that certain transcription factors related to the oestrogen signalling cascade will be involved in

endometriosis and we will look to investigate those transcription factors further.

During my research (as explained in Chapter 4:Data and Analysis), FOXD3 was the transcription factor we identified to be the key player in the pathogenesis of patients with endometriosis.

1.7.1-What is FOXD3?

FoxD3 is a transcription factor belonging to the fork-head family of transcription factors (characterized by a fork-head box domain for binding). The transcription factor binds to the sequence 5'-A[AT]T[AG]TTTGTTT-3' and acts as a transcriptional activator and repressor. FOXD3 is known to promote development of neural crest cells from neural tube progenitors. (105) They are important in promoting the development of neural crest cells, restricting neural progenitors cells to the neural crest cells. FOXD3 is important in the maintenance of pluripotent cells in the peri and pre implantation stages of embryogenesis, which is the process in which the embryo forms and develops into the foetus. (105)

Currently there has been no investigation in human or animal studies of the role of FOXD3 in endometriosis. In referring to Chapter 3: Data and Analysis, it was found that FOXD3 regulates many genes that are involved in endometriosis.

Therefore in the process of the study described in this thesis, I collated available evidence on a variety of cellular functional aberrations associated with endometriosis; employed bioinformatics tools to systematically investigate the interaction between different cellular biological pathways and predicted key players including the transcription factor FOXD3. In a brief laboratory experiment I tested the involvement of this Transcription factor in patient samples and confirmed its possible involvement in endometriosis. Therefore I propose that the use of bioinformatics in this way is a fundamental advancement to improve understanding of the pathological process of endometriosis and further studies are needed to identify key therapeutic and

diagnostic targets with the use of bioinformatics tools before actual studies on human subjects. This approach will reduce the need for unnecessary invasive procedures on animals and in humans.

Our study was able to identify the common genes, proteins and transcription factors, which were key players in processes such as apoptosis, calcium signalling, proliferation and senescence of the endometrium. Our main finding has been experimentally validated and suggests differential role of FoxD3 and the associated transcription factors in endometriosis. We were able to confirm the presence of the main transcription factor FOXD3 in endometrial samples of normal fertile control patients, and examine if aberrant expression of FOXD3 (and the genes its regulates) in patients with endometriosis was able to correspond to our bioinformatics findings.

CHAPTER 2

2- BIOINFORMATICS METHODOLOGY

From what was discussed in the introduction, a bioinformatics approach was used to investigate the genes and proteins involved in the pathological processes within endometriosis, and how they may be inter-linked with each other.

Bioinformatics is a field of molecular and genetic biology that is being brought to the forefront of today's scientific research. An increasing amount of research projects today have integrated bioinformatics and microarray studies into their work when investigating various conditions and biological pathways.

When PubMed was used and the keywords "bioinformatics endometriosis" was inserted, the search returned with 41 related studies. Numerous studies in this search investigated various biomarkers, which are used as diagnostic tests for patients with endometriosis. For example, Tokushige et al used bioinformatics to study the proteins that were found in urine of endometriosis patients. (106) In a similar way to Tokushige et al in his study, Ferrero et al used 2-D gel electrophoresis and a bioinformatics approach to carry out a proteomic analysis of the peritoneal fluid of women in endometriosis. (107) However, these studies and 2 others identified in the literature search do not provide a genetic explanation into the pathology and aetiology of endometriosis comprehensively. In contrast, our study identifies the common biological processes associated with endometriosis. We will also identify common genes and transcription factors that are affected in patients with endometriosis.

Rai et al in 2010 used bioinformatics to study the proteome profiling of the endometrium in women with endometriosis. (108) Proteomics (study of proteins) were used to compare protein expression in the endometrium of patients with endometriosis to normal fertile control patients. The study was

successful in identifying genes, which were responsible for endometriosis. (108) Rai et al identified the dysregulation of more than 70 proteins in the proliferative phase of eutopic endometrium in stage 4 of endometriosis and secretory phase of stages 2,3 and 4 endometriosis. The study found other key genes that were up regulated in patients, such as DJ-1, HSP27, HSP60, HSP70, GRP78, HSP90 beta, and MVP. (11) In using a bioinformatics approach to identify the proteomic changes in the endometrium of women with endometriosis, the study failed to identify how related common biological processes and pathways may be affected.

Our study was able to identify the common genes, proteins and transcription factors, which were key players in processes such as apoptosis, calcium signalling, proliferation and senescence of the endometrium. Our study progressed further and used laboratory techniques to examine how the common transcription factors (involved in regulating common key-genes) are affected in patients with endometriosis.

2.1- Endometriosis Genetic data mining

In order to collate the genes and proteins that may be linked to the various pathological processes occurring in endometriosis, we carried out comprehensive PubMed searches for journals that have studied such processes.

To gain an initial understanding, we conducted a search using the keywords “Endometriosis bioinformatics” and “Endometriosis microarray studies”. This helped us view the common genes that are expressed in patients with endometriosis. The studies also provided us an insight into genes and proteins that have been investigated as being possible diagnostic markers in patients, or genes that are up/down regulated upon treatment in patients. We then progressed to conducting separate searches of different biological or pathological processes involved in endometriosis. Each search was completed separately to find and review journals, which contained information

on genes and proteins implemented in the disease. The specific searches conducted provided specificity to our genetic and molecular data we collated allowing for organization when we inputted them into various computational software.

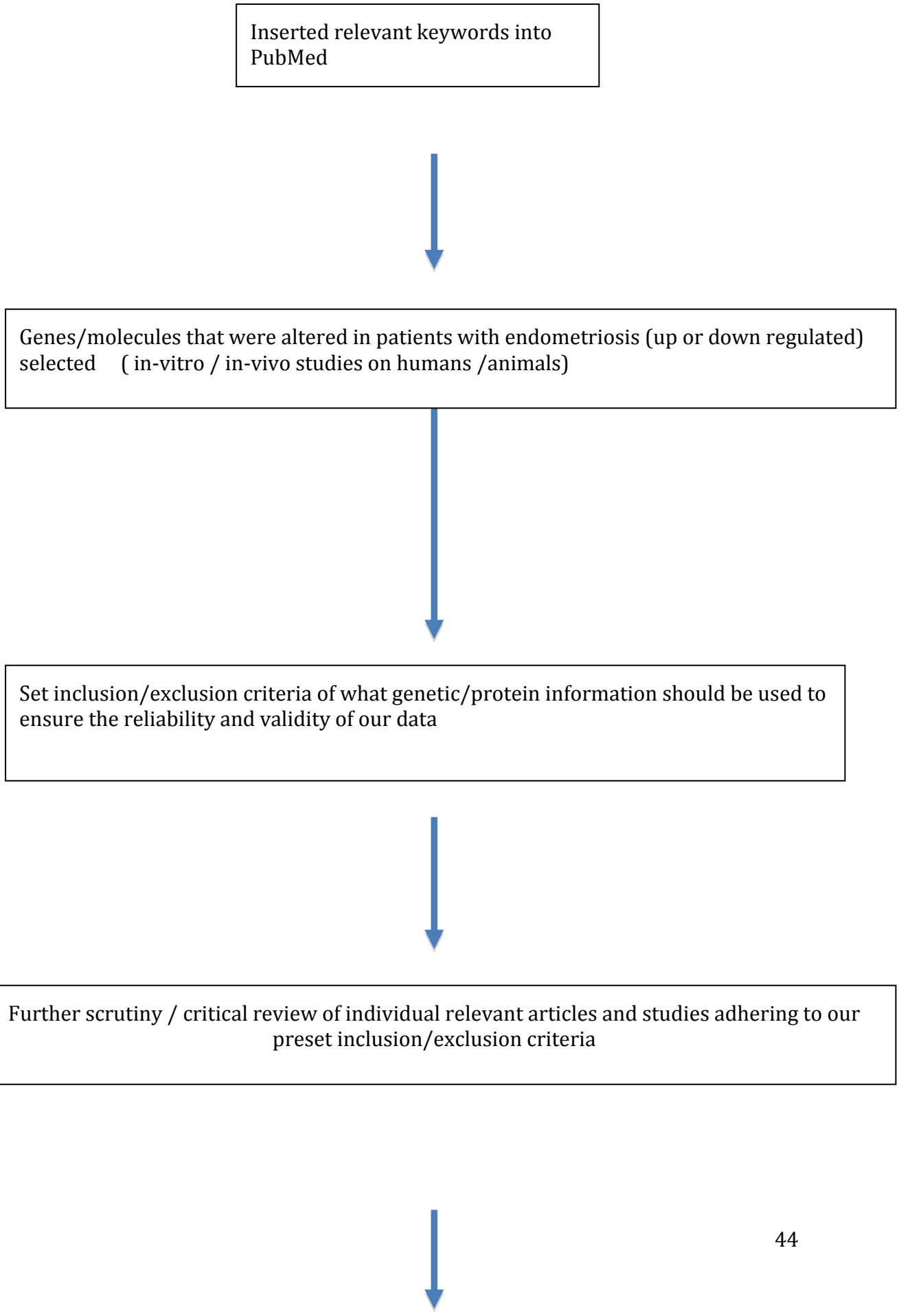
Therefore, as discussed in the introduction, it is important different keywords were inputted into PubMed to investigate each pathological process.

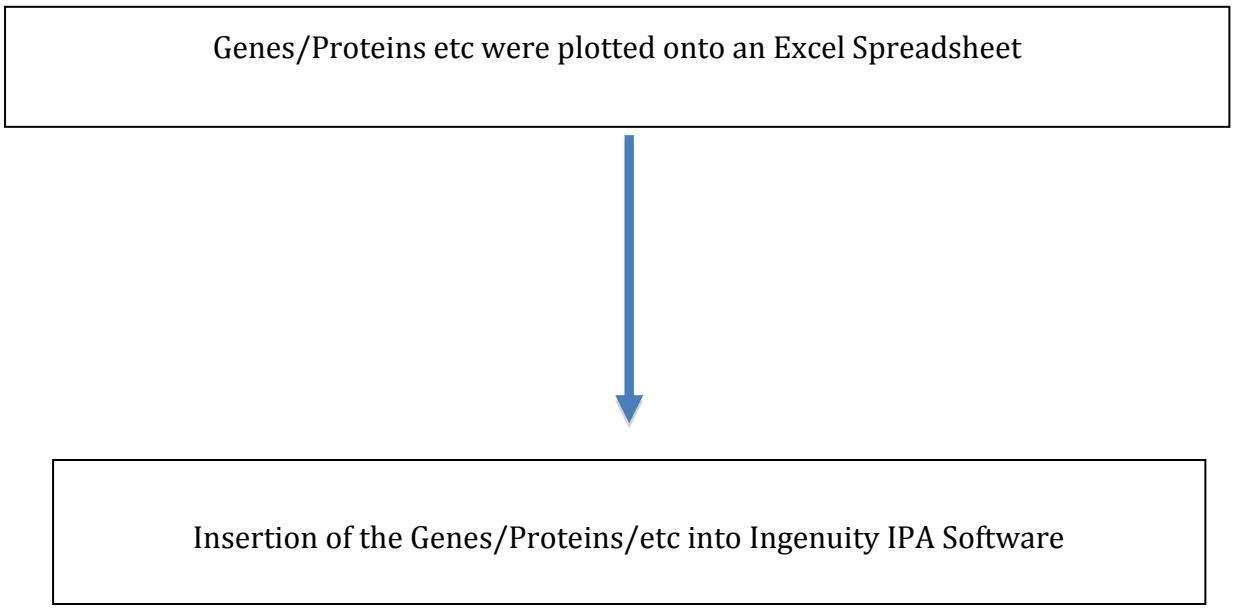
Endometriosis microarray studies- returned with 67 studies
Senescence Endometriosis- returned with 4082 studies
Proliferation Endometriosis- returned with 545 studies
Oestrogen responsiveness Endometriosis- returned with 27 studies
Embryo Implantation Endometriosis- returned with 224 studies
Embryo implantation in Endometrium (CONTROL SEARCH)- returned with 2838 studies
Inflammation Endometriosis- returned with 422 studies
Altered Immune Response Endometriosis- returned with 17 studies
Retrograde Menstruation Endometriosis- returned with 1080 studies
Metastasis Endometriosis- returned with 266 studies
Calcium signalling Endometriosis- returned with 2 studies
Progesterone Endometriosis- returned with 1175 studies
Prostaglandins Endometriosis- returned with 197 studies
P450 Endometriosis- returned with 57 studies
Apoptosis Endometriosis- returned with 243 studies.

We used the keywords “embryo implantation in the endometrium” to compare the difference in gene expression in the process of implantation in the normal endometrium to that of patients in endometriosis, if it was found that any genes would be aberrantly expressed.

There have been many studies, such as Salamonsen et al in 2002 that have shown molecules such as calbindin-D9k, a regulator of calcium, to be up regulated by progesterone and increased in the uterus during early pregnancy. (109) Also other cytokines such as MNSFbeta were shown to have a lower expression in implantation sites on day 4 and 5 of pregnancy upon the embryo attaching in endometriosis patients. (6) A selection of many other molecules such as hsa-miR-101, hsa-miR-144, and hsa-miR-199a have been shown in studies such as that by Chakrabarty et al in 2007 to target the COX-2 gene, which was involved in peri and pre-implantation of the embryo in mice studies. (110)

2.2-Overview of Gene/Protein selection from the Literature





2.2.1- Inclusion/Exclusion criteria of genetic selection

We looked to select genes/molecules from research articles that have conducted in-vitro and in-vivo experimental work when investigating expression profiling in patients with endometriosis. We excluded genes/molecules expressed in endometriosis according to review articles because no individual lab work was conducted in these studies, so reliability of the data could be questioned.

We reviewed studies that conducted work on both animal and human specimens. Studies were also included that had been conducted *in vitro*. An *in-vitro* study refers to one that is conducted on an organism that has been isolated from their biological context. (111)

The advantage of using studies that have conducted *in-vitro* work is that investigators have been able to obtain data from living organisms in which biological pathways and processes are fully functional. (111) We were able to obtain details on genes/molecules that have been investigated in various interactions, pathways and processes in a living organism. Common studies often include cells derived from multicellular organisms such as a cell or tissue culture or purified molecules in a test tube. (2) Examples include Polymerase Chain reaction, in-vitro fertilization and protein purification.

We critically reviewed each article to ensure any genetic/molecular marker we used from the study possessed reliability and validity. We ensured each study was conducted in the past 10 years and preferably no later. With advances in technology, findings related to endometriosis may be different now compared to 20 years ago, even though still little is known regarding the pathogenesis and etiology of the disease currently. We also made sure the study was conducted in a suitable institute such as in a University department or in an Obstetrics and Gynecology department of a hospital. The setting of a study displays up-to-date technology and laboratory equipment is used, as well as appropriate samples for the study (samples from patients with endometriosis and relevant controls).

We also ensured the study had obtained its genetic data from a sufficient sample size. There are many studies that have conducted in-vitro studies on small sample sizes, such as a small patient group (less than 10 patients). This presents an issue of the data not being of credible nature. It was also important for the study to indicate where the samples had been collected from and how they had been collected before they were studied in an in-vitro manner. Noting that the samples had been collected from hysterectomies, endometrial pipelle procedures or obtaining cells from peritoneal fluids from patients are some examples of suitable procedures.

It was also important for us to be aware asto how the patients for sample collection had been selected. For much of our search categories, such as calcium signaling, proliferative activity in patients with endometriosis and

others, different unrelated conditions can affect the genes and proteins related to the these pathological and biological processes that were searched. Therefore, it was important the studies had used samples in which the patients had no other conditions or were taking no medication at the time. It was also important that the studies used samples from patients who weren't pregnant or had not had past recurrent miscarriages. Studies had to use samples collected from patients of the same age and demographic groups.

We obtained genes and proteins expressed in the various biological and pathological processes of interest in only patients with endometriosis. From the different studies, these genes and proteins were compiled into a table under the different categories. We also looked for names of important receptors that were involved in the processes of interest (e.g receptors involved in calcium signaling, proliferation etc).

The tables of genes/molecules were then managed in the following manner using the appropriate programs. These programs were helpful in providing any link between genes/molecules/proteins differentially expressed at the different biological processes, and also identifying the regulation or pattern of expression of these particular genes.

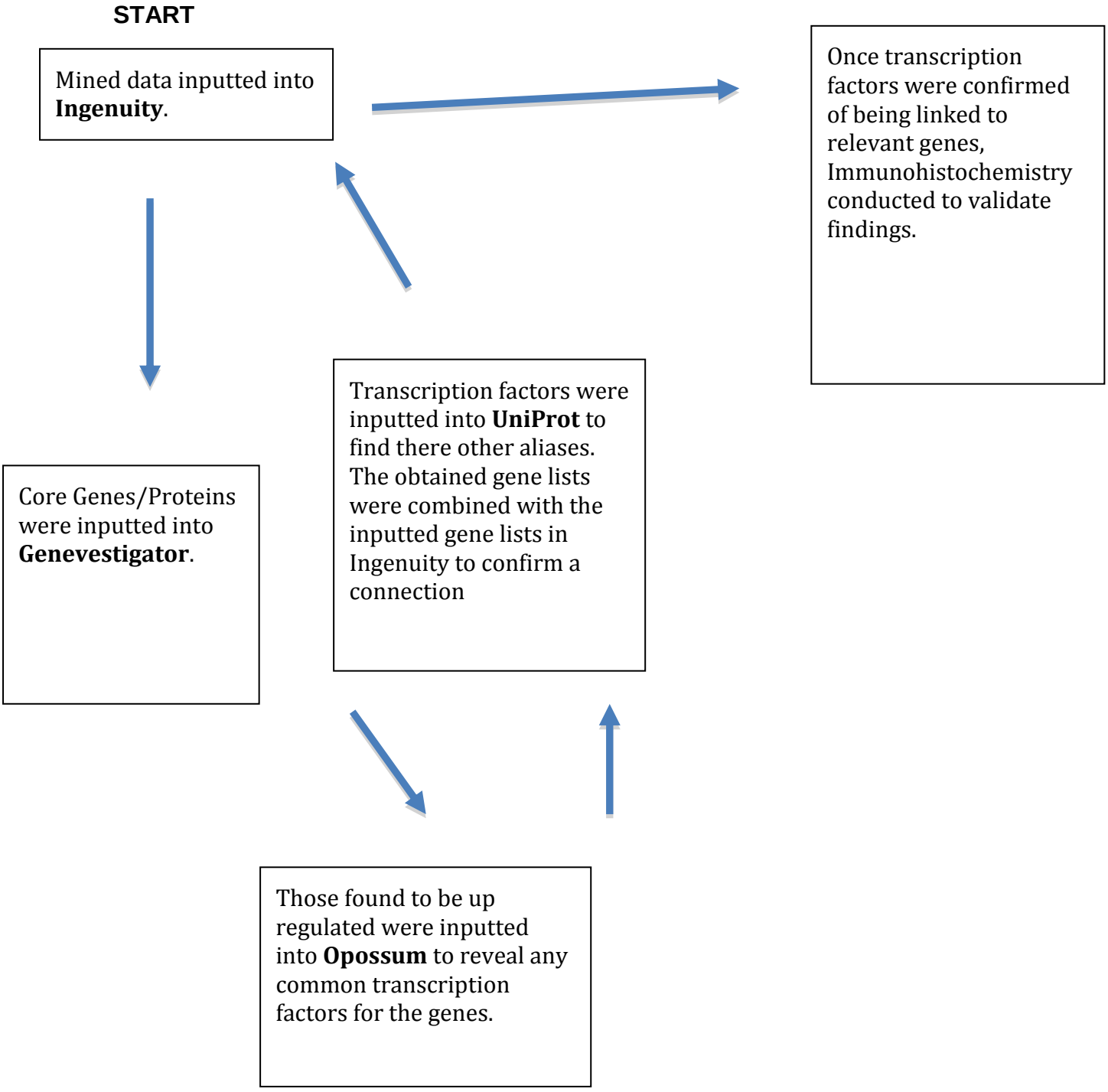
2.2.2- Data Mining Output

Below is an example of a list of genes that was formulated from one of the keyword searches we inputted as part of our Data mining search on "Proliferation Endometriosis". This data was found from the study to be relevant to endometriosis during the proliferative activity in the endometrium.

15-PGDH	5-LO	MCP1	IL-6	BRCA1	FKBP52	FR 167653	PPAR-bp	PAR1
COX-2	TLR-4	COL4A2	PGE2	BRCA2	IL-10	HLA-G	GRO1	PPACK

IL-8	NF-κB	COL5A2	S100-A8	GnRH	CCL21	MCP-1	GRO2	ALDH

2,3- Overview Bioinformatics Management of Data



2.3.1- The use of Ingenuity IPA Software

Ingenuity IPA is a software program, which allows research projects to analyze and gain an understanding of biological pathways in science. IPA integrates data from different experiments to provide insights into biological and chemical interaction in common pathways as well as the progression of a disease in these canonical pathways and other systems. IPA is an important tool for drug discovery as well as gene and protein discovery. Founded in 1998, Ingenuity is an important driving force for a bioinformatics approach in the scientific research world. The ability of the program to visualize, explore and analyze biological experimental data allows insightful work for researchers part of the pharmaceutical and academic institutes.

The program allows efficient analysis and interpretation of genomic and proteomic datasets for profiling, which helps many researchers and organizations identify therapeutic targets and connection. The program also provides an understanding into the toxicity of a drug, as well as identifying important biomarkers through gene expression and proteomic array studies.

INPUT OF DATA MINING GENES AND MOLECULES FROM LITERATURE SEARCH

Figure 2.3.1.1 (found at the end of this chapter with corresponding figures from this chapter) is an example of the input of genes and molecules found from the above literature search on “proliferation endometriosis” into Ingenuity. Ingenuity was able to translate the inputted raw data and provide the full names of genes and proteins in this figure, as well as the location in the cell.

OUTPUT

Figure 2.3.1.2 shows how Ingenuity IPA instantly translated the data spreadsheet and provided a list of genes that are differentially expressed in endometriosis from an illustrated interlinked map of figures involved in proliferation. This was accessed from Data Analysis>Networks>Merge all available networks>Overlay functions>Functions and Diseases> Reproductive System Disorder

The next stage was to input this data into Genevestigator to investigate, which genes were up regulated and which were down regulated.

2.3.2-The Use of Genevestigator V3

Genevestigator is a software program, which shows genes that are up regulated and down regulated in certain conditions. Genevestigator V3 provides a meta-analysis of gene expression across a collection of experimental microarray data. (112) The Meta analysis provides a confirmation of our findings from Ingenuity, and greater insight into the activities of specific genes from the list expressed in endometriosis. (112)

Through a Hierarchical Clustering, the tool allows the group of genes with similar profiles across arrays to be clustered and shown to be up/down regulated in conditions or various anatomical parts of the human body.

INPUT OF GENETIC/MOLECULAR INFORMATION

Below is a list of genes involved in proliferation of the endometrium differentially expressed within the endometriosis according to Ingenuity.

BCL2	SMAD6
CYP19A1	CCR1
IL8	FPR2
ROCK2	NRSA1
BRCA1	CDK1

DYNLL1	GREM1
NFKB1	PIK3R1

We would input these into the Meta analyses program to find which were up regulated in endometriosis. Figure 2.3.2.1 shows the initial screen in which raw data is inputted. The array selection for the genes to be analyzed was from Human Genome studies only (as opposed to mouse or animal studies).

OUTPUT OF GENES UPREGULATED IN ENDOMETRIOSIS

Those genes coded indicated in red in Figure 2.3.2.2 were be up regulated according to the meta-analysis.

It was found BCL2, IL8 AND SMAD6 were up regulated.

The next step would be to input these 3 up-regulated genes into Opossum to discover which is the common regulatory transcription factor.

2.3.3- The Use of Opossum

Opossum is a web-based program useful for detecting transcription factor binding sites in sets of co-expressed genes. Opossum is an important system that searches for evidence of co-regulation of genes (or a group of genes) by a common transcription factor. The program also identifies, through a pre-combined computated database, conserved transcription factor binding sites of these transcription factors for the genes that are regulated. (113) Opossum has been important in providing identification of common transcription factors in human studies, as previous work on human genomic regulatory studies were ineffective.

INPUT OF UPREGULATED GENES INTO OPOSSUM

The web-based program is used from the input of the genes of interest into the site as shown in Figure 2.3.3.1. Human studies are then selected, as well as the option of ‘HUGO/MGI Symbol/Alias’ for the GeneID type. Taxonomic

super groups can also be selected (plant, human or insects) as well as the amount of upstream/downstream sequences the program wishes to produce. We selected 2000/0 on the upstream/downstream score to provide a concise enough list of relevant transcription factors regulating the genes as close to the promoter region as possible. The selection criteria can be seen in the screen shot above.

The ordering of transcription factors produced was sorted by a Z-Score. The electronic Z score expresses the divergence of the value so the larger the Z score, the less probable the result will be due to chance.

OUTPUT OF DESIRED TRANSCRIPTION FACTORS

From the group of transcription factors, it was found ZNF354C to be the transcription factor which had the most background hits and most binding sites for genes related to proliferation that were up regulated in endometriosis which we inputted. The final stage would have been to use UniProt to find any alternative names for this transcription factor. As can be seen in Chapter 3: Data and Analysis Chapter, FOXD3 was the most common transcription factor found to regulate a majority of the genes found up regulated in endometriosis. Therefore, the final step was to input FOXD3 into UniProt.

2.3.4- The Use of UNIPROT

UniProt is a web search based program that uses various databases to provide different sequence names for one specific protein/molecule inputted. The databases used are Uni Prot Knowledgebase, UniProt Reference Clusters and UniProt Archive. The program is also used for aligning protein sequences together as well as identifying proteins in a specific sequence put into the program.

Figure 2.3.4.1 shows how UniProt was used. With FOXD3 (well-known transcription factor) being inputted into the Protein Knowledgebase, the search returned with 11 different other accessions of the transcription factor FOXD3. From here, we used FOXD3 and its human accessions and put this back into Ingenuity with the respective genes that it regulates.

Immunohistochemistry (IHC) was used to validate our bioinformatics findings as seen in Chapter 3: Data and Analysis. We were able to confirm the presence of the main transcription factor FOXD3 in endometrial samples of normal fertile control patients, and examine if the aberrant expression of FOXD3 (and the genes its regulates) in patients with endometriosis was able to correspond to our bioinformatics findings.

2.4-IMMUNOHISTOCHEMISTRY (IHC) METHODOLOGY

Immunohistochemistry Introduction

Immunohistochemistry is an important procedure for detecting the expression of particular antigens in a tissue or cell by observing the binding of antibodies to the targeted antigens. (114)

The procedure is important in research such as this, in visualizing how biomarkers and specific proteins may be localized and distributed in certain tissues at a cellular level. (114) It is useful for the diagnosis of abnormal (cancer cells) in patients through a clinical setting. An antibody-antigen interaction can be visualized a number of different ways, including the antibody being conjugated to an enzyme such as peroxidase or the antibody being tagged to a fluorophore which allows immunofluorescence detection. (115)

An antibody (Ig) is a glycoprotein used by the immune system to identify foreign substances such as bacteria and viruses (antigens) and neutralize them. (114) There are five classes of antibodies: IgG, IgA, IgM, IgE and IgD. In Immunoassays, IgG and IgM are important, Ig G, the most abundant antibody, is produced by B Lymphocytes and provides antibody-based immunity against antigens. The classical “Y” shape of the IgG molecule (MW ~150 kD) is composed of four polypeptide chains — two light chains (each with a molecular weight of ~25 kD), and two heavy chains (each with a molecular weight of ~50 kD) connected with a disulphide bond. (114) Each end of the Y portion of antibody is called the Fab region (fragment antigen

binding region). The Fab and Fc regions are useful in immunoassays, with antibodies being labelled in the Fc region during immunohistochemistry. (114)

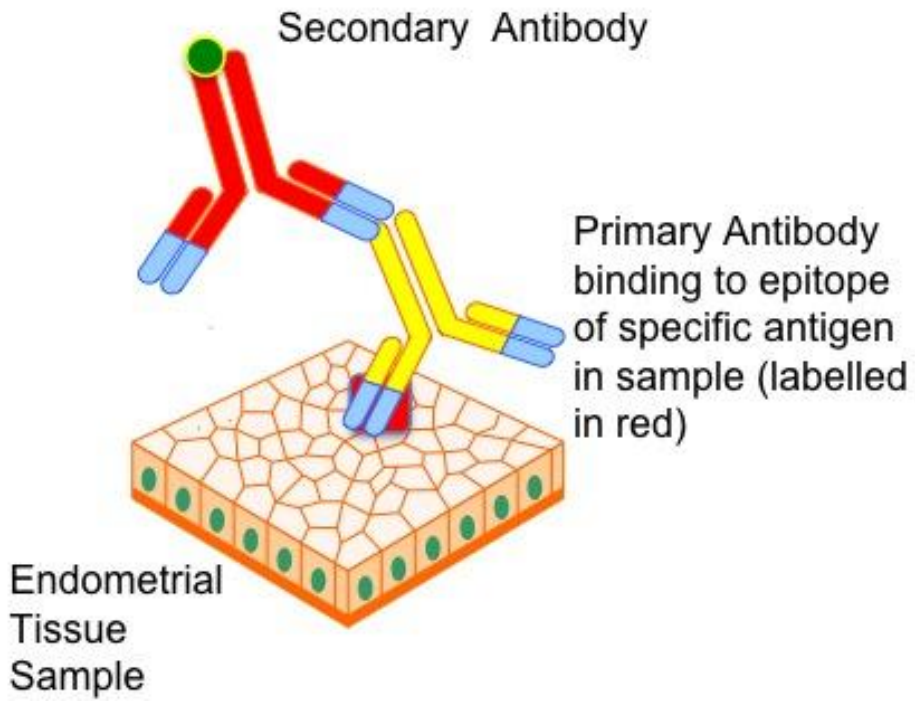
Antibodies used for detection may be either polyclonal or monoclonal antibodies. In our laboratory experiments, we used both in the staining runs. Polyclonal antibodies are those produced by immunizing animals such as rabbits. The serum contains antibodies derived from different types of immune cells and hence is known as polyclonal. (116) Rabbit polyclonal antibodies to Foxd3 were used in our experiment.

Monoclonal antibodies are those produced from a clone (single cell line). Replicable Tumor cells are fused with immunized mammal cells, such as that of a mouse, which produce antibodies. (116)

Polyclonal antibodies are effective, such as Rabbit IgG, in having the affinity to bind to numerous antigen receptors. Therefore FOXD3 was raised in the polyclonal antibody as it interacts with many different epitopes (of various genes/proteins). Monoclonal antibodies show specificity for a single epitope, therefore displaying a greater specificity than polyclonal antibodies. Therefore, this is the reason we used Progesterone and androgen receptors raised from mouse.
In order to visualize the localization of the antibody binding, an appropriately labeled secondary antibody is used in conjunction with the primary antibody. For example, if the primary antibody was raised in the mouse, an anti-mouse secondary antibody will be used, and if the primary antibody is raised in a rabbit, the secondary antibody will be anti-rabbit. In our lab work, the polyclonal rabbit IgG was used as a primary antibody and the secondary antibody used was the ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer. The ImmPRESS Anti-Mouse Ig (peroxidase) polymer was used as a secondary antibody when the primary antibody was mouse IgG being in the staining of Androgen and Progesterone receptor in endometrial cells. The peroxidase is a rapid and stable enzyme and the HRP conjugate is an affinity-based antibody and reacts with the light and heavy chains of the rabbit IgG or mouse IgG.

The secondary antibody was specific therefore to the primary antibody. This method is known as an indirect immunostaining method and is more specific to a direct staining method (only primary antibody binding to the antigen) as the signal is amplified. (116) The signal for staining is amplified as numerous secondary antibodies binds to the primary antibody through the Fab and Fc fragments. (116)

An image showing the Illustration of antibody-antigen interaction during immunohistochemistry.



Above is an illustration of how the antibody and antigen interact. As mentioned before, the primary antibody (for example the FOXD3 raised in rabbit) binds via its Fab and Fc fragments to the epitope of the antigen. The secondary antibody will bind to the primary antibody and the substrate stain (illustrated as a blue circle) would bind to the secondary antibody allowing for visibility microscopically.

During the immunohistochemistry, in both staining of the FOXD3 and Progesterone Receptor and Androgen Receptor, we used tissue from the

endometrium of women with recurrent miscarriages, and the placenta as a positive control. Both were known to stain upon the addition of both antibodies.
As a comparison to looking at the FOXD3 staining (primary polyclonal antibody raised in rabbit) in our samples, we also used normal polyclonal rabbit IgG on the samples, to ensure specificity of the staining. A negative staining was expected from normal rabbit IgG antibodies on the sample cells and therefore acted as a negative control.
Similarly, when looking at the staining of Progesterone and Androgen receptors in our samples, which were both raised in mouse, normal monoclonal mouse IgG was added to the samples again to show specificity.

Other control measures that were taken were using a serum blocker when we used the polyclonal rabbit IgG antibody. This was added after the primary antibody had been added to the slide. Serum blocker ensures all epitopes on the tissue sample were blocked to prevent any nonspecific binding of the antibody, improving sensitivity of the assay. (116)

2.4.1- The use of IHC to detect FOXD3

Our findings from the bioinformatics study had shown how the transcription factor FoxD3 was the key factor involved in regulating the expression of up regulated genes involved in the pathology of endometriosis. We subsequently tested the hypothesis we generated from our bioinformatics research, and conducted immunohistochemistry experiments to observe the interaction between FOXD3 antibodies and genes that are expressed in endometriosis. We also observed microscopically the interactions of progesterone and androgen receptor antibodies and genes expressed in patients with endometriosis. This was based on our bioinformatics research in which we found genes related to progesterone and androgen receptors being expressed in endometriosis according to Ingenuity and Genevestigator. It was also found that FOXD3 regulated genes involved in the expression of progesterone and androgen receptors such as estrogen receptors.

For our immunohistochemistry work, we used 20 patient samples in total (n=20), with 10 patients having active peritoneal endometriosis and 10 normal fertile control patients.

2.4.2- Ethical Approval

A literature review was previously performed to ensure any work conducted in this study provides novel scientific information. Ethical approval was obtained for the study from the Liverpool Regional Ethics Committee (LREC) (09/H1005/55) and patients were recruited at the Liverpool Womens Hospital. All participants included had given informed written consent.

2.4.3- Selection of Patient Samples

All patients were consented in a confidential manner, having being briefed on the purposes of the use the samples for the research during the consenting.

Biopsies were taken from fertile control groups and an endometriosis group.

Endometrial biopsies were collected at the luteal and follicular phase of the menstrual cycle. The follicular phase (proliferative phase) is the phase in the menstrual cycle where the ovaries mature. Rising oestrogen production during the follicular phase allows for growth of the endometrium in the uterus, and progesterone receptors are produced in endometrial cells so the endometrium is ready for the rising progesterone level during the luteal phase. The luteal phase (or the secretory phase) is the later phase of the menstrual cycle. Progesterone is produced in this phase, and is important in making the endometrium receptive to the implantation of the blastocyst (window of implantation).

The samples used in our experiments were endometrial pipelle and Full thickness samples collected from the patients. Pipelle samples are those collected from endometrial biopsies and only contain the functional layer,

whereas full thickness endometrial samples possessed the basal and functional layer of the endometrium. Full thickness samples are those cut from the endometrium of the uterus surgically removed from the patient during a hysterectomy. 11 samples out of the 20 collected were full thickness specimens.

2.4.4- Inclusion/Exclusion Criteria of Patients Selected

Reproductive women with regular menstrual periods were included in the study. Patients seen to have had taken regular medication, been on hormonal treatment or breast-feeding in the past 3 months were excluded from the study. Also, patients with an intrauterine or Mirena device were excluded, as well as those patients who have had a regular miscarriage or any recent diagnosis of cancer. Those who had exhibited abnormal bleeding, or have displayed symptoms for any other unrelated condition were excluded.
Patients were included in the endometriosis study group provided they have had active peritoneal endometrial and complained of major symptoms (these symptoms being listed in the Chapter 1: Introduction).
Normal fertile patients must have had at least 1 pregnancy and must not have had any endometrial pathology or symptoms related to endometriosis such as pelvic pain. All normal women must have had their pelvis assessed. This is be done through an investigative procedure known as a laparoscopy. (25)

Sample Processing / Tissue sectioning

Once collected, the samples were stored in NBF formalin at room temperature. The tissue samples for experimental work were then embedded onto wax blocks, cut and appropriately coated. They were labelled with the specimen ID, date of the staining as well the details of the antibody employed for that slide (FOXD3 1in X)

2.4.5- Immunohistochemistry procedure

De-waxing of the sample

The prepared sectioned slides were submerged into xylene and differing concentrations of ethanol to completely dewax the samples on the slides in the rack.

Antigen retrieval stage of the samples

A heated 10mM citrate buffer solution was prepared, in which the slides were submerged in for 1 minute to break protein bonds and reveal antigenic sites for antibody interaction. (114)

The slides were then placed into a 0.3% dilution of hydrogen peroxide (H202) in TBS solution for 10 minutes. The area of interest on the slide was then circled with hydrophobic pen marker.

Addition of antibodies to the sample

50microlitres of the respective antibody was added to each section. Following this, a drop of the respective secondary antibody was added (explained earlier in chapter). Below is a table of concentrations used in each of the staining runs we completed.

Antibody	Concentration	Source	Incubation period
FOXD3 (Polyclonal antibody in rabbit)	1:800	BioLegend Ltd	After addition of antibody & serum blocker, left overnight at 4 degrees centigrade to ensure optimum interaction.
Rabbit IgG (rIgG)	1:4000	BioLegend Ltd	
Progesterone Receptor (PR) (Monoclonal antibody raised in mouse)	1:100	BioLegend Ltd	After addition of antibody, PR, AR and MIgG slides left for 30 minutes room

			temperatures to ensure optimum interaction
Androgen Receptor (PR) (Monoclonal antibody raised in mouse)	1:50	BioLegend Ltd	
Mouse IgG (mIgG)	1:4000	BioLegend Ltd	

Addition of substrate staining

After the addition of the secondary antibody, a drop of the substrate DAB solution (brown deposits to the cell) were added to each slide and left at room temperature for 10 minutes. Following this, the slide rack was dipped in Haematoxylin, a counterstain used to stain the nucleus blue, allowing for the antibody-antigen interactions to be analyzed.

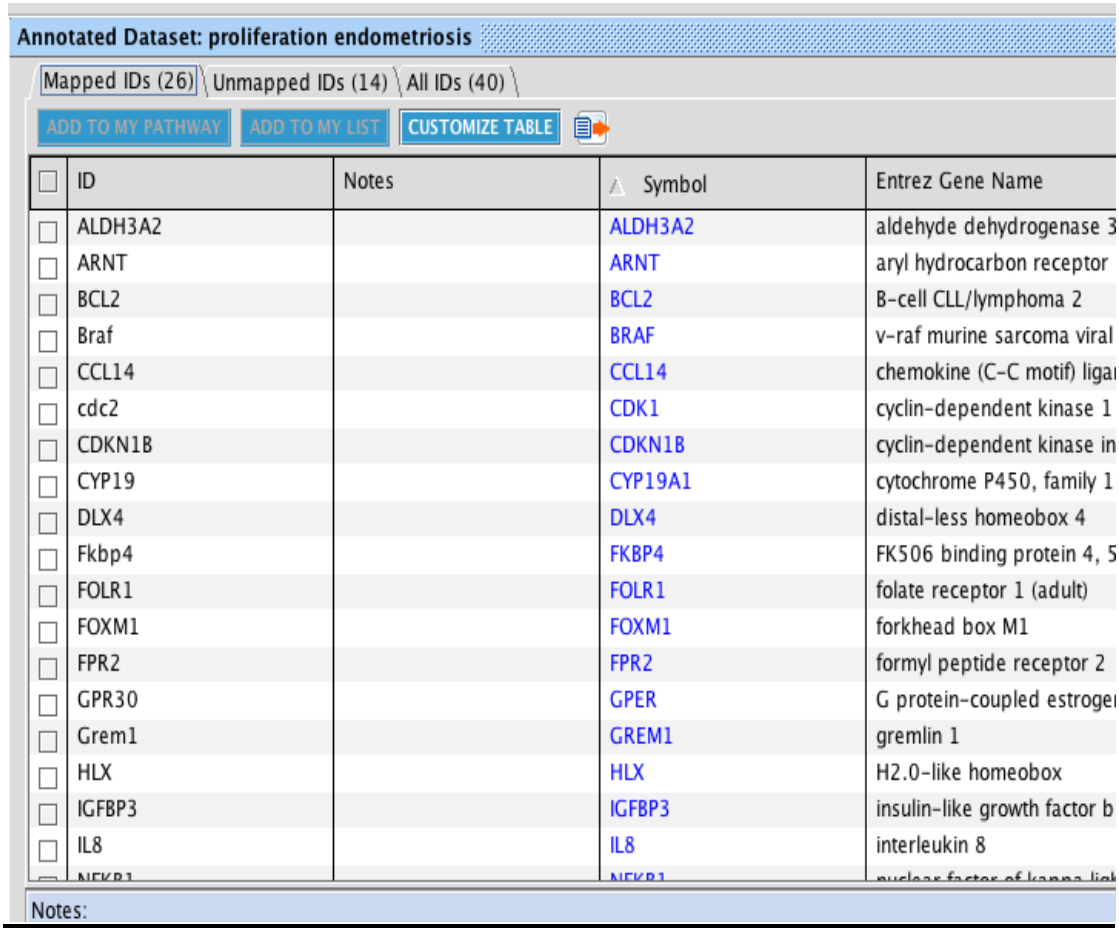
Dehydration of Slides

After the slides had been dipped in acid alcohol and placed under running water to cease substrate reaction, they were dehydrated with differing concentrations of ethanol and xylene. This allowed for the slides to then be mounted and analyzed microscopically.

The intensity staining shown on the slides was subsequently analyzed from microscopically scoring each slide, and inputting the data into the statistical program SPSS. The data and analysis of the bioinformatics findings being validated through IHC work can be seen in the next chapter.

CHAPTER 2 FIGURES

FIGURE 2.3.1.1



Annotated Dataset: proliferation endometriosis

Mapped IDs (26) | Unmapped IDs (14) | All IDs (40)

ADD TO MY PATHWAY | ADD TO MY LIST | CUSTOMIZE TABLE

ID	Notes	Symbol	Entrez Gene Name
ALDH3A2		ALDH3A2	aldehyde dehydrogenase 3
ARNT		ARNT	aryl hydrocarbon receptor
BCL2		BCL2	B-cell CLL/lymphoma 2
Braf		BRAF	v-raf murine sarcoma viral
CCL14		CCL14	chemokine (C-C motif) liga
cdc2		CDK1	cyclin-dependent kinase 1
CDKN1B		CDKN1B	cyclin-dependent kinase in
CYP19		CYP19A1	cytochrome P450, family 1
DLX4		DLX4	distal-less homeobox 4
Fkbp4		FKBP4	FK506 binding protein 4, 5
FOLR1		FOLR1	folate receptor 1 (adult)
FOXM1		FOXM1	forkhead box M1
FPR2		FPR2	formyl peptide receptor 2
GPR30		GPER	G protein-coupled estroge
Grem1		GREM1	gremlin 1
HLX		HLX	H2.0-like homeobox
IGFBP3		IGFBP3	insulin-like growth factor b
IL8		IL8	interleukin 8

Notes:

FIGURE 2.3.1.2

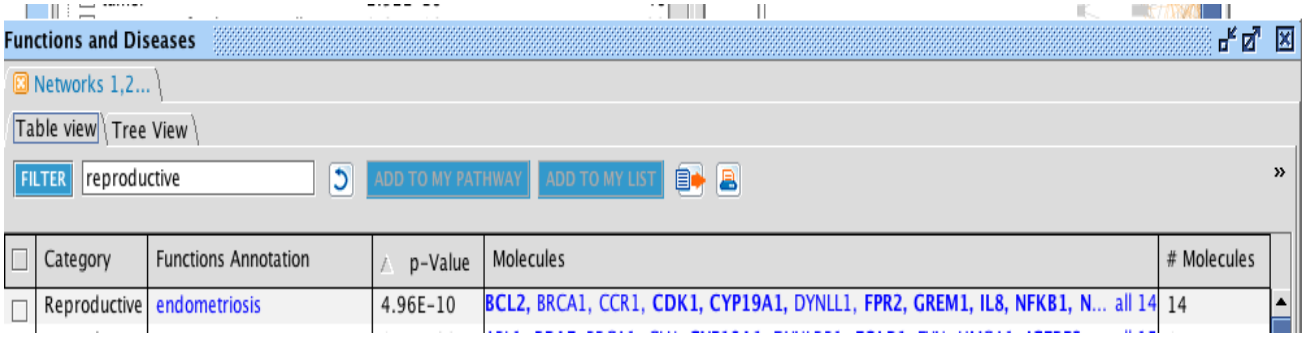

Functions and Diseases
Networks 1,2...
Table view
Tree View
FILTER
reproductive
ADD TO MY PATHWAY
ADD TO MY LIST
Category
Functions Annotation
p-Value
Molecules
Molecules
Reproductive
endometriosis
4.96E-10
BCL2, BRCA1, CCR1, CDK1, CYP19A1, DYNLL1, FPR2, GREM1, IL8, NFKB1, N... all 14
14

FIGURE 2.3.2.1

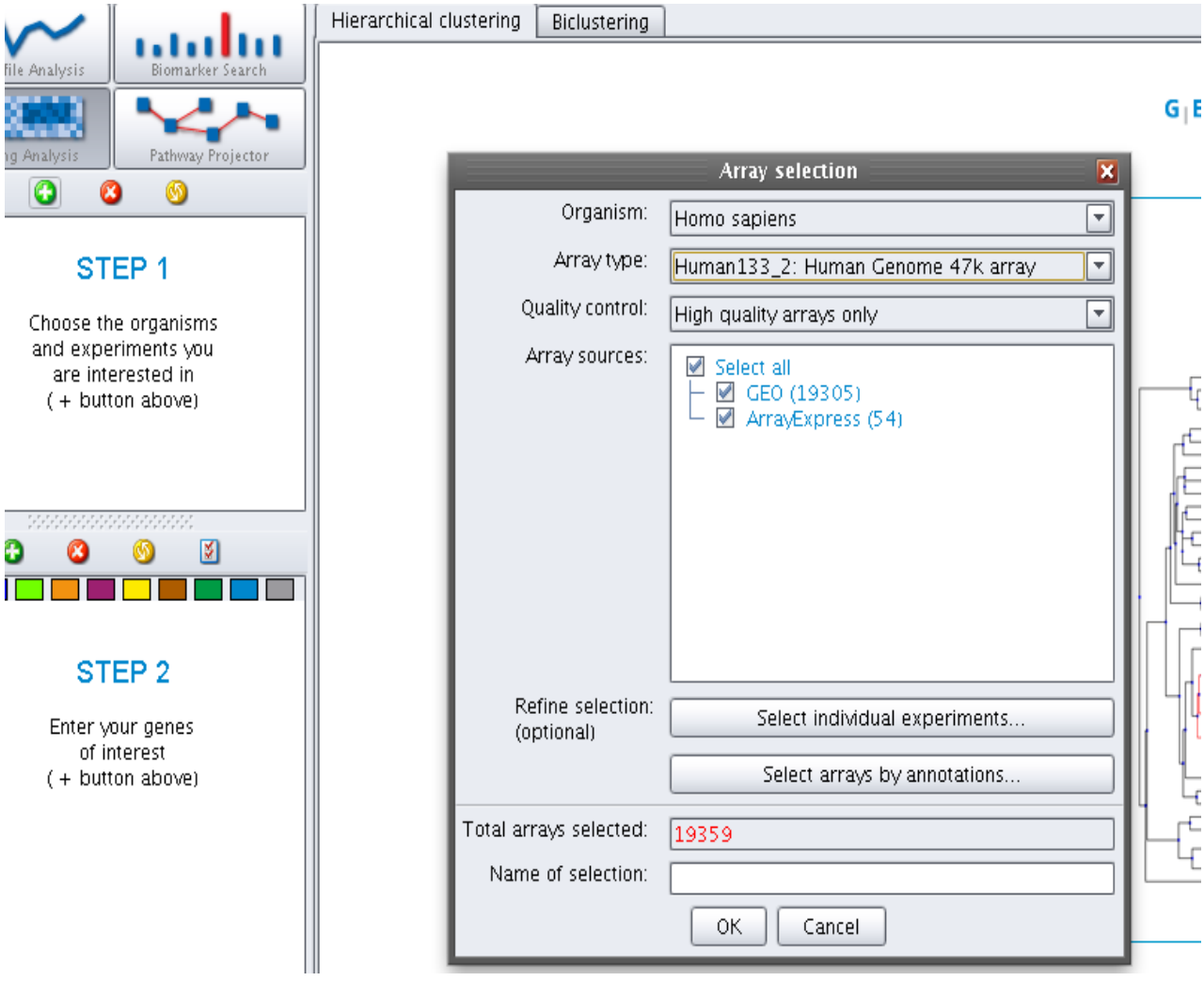

Hierarchical clustering
Biclustering
Biomarker Search
Pathway Projector
STEP 1
Choose the organisms and experiments you are interested in (+ button above)
STEP 2
Enter your genes of interest (+ button above)
Array selection
Organism:
Homo sapiens
Array type:
Human133_2: Human Genome 47k array
Quality control:
High quality arrays only
Array sources:
Select all
GEO (19305)
ArrayExpress (54)
Refine selection: (optional)
Select individual experiments...
Select arrays by annotations...
Total arrays selected:
19359
Name of selection:
OK
Cancel

FIGURE 2.3.2.2

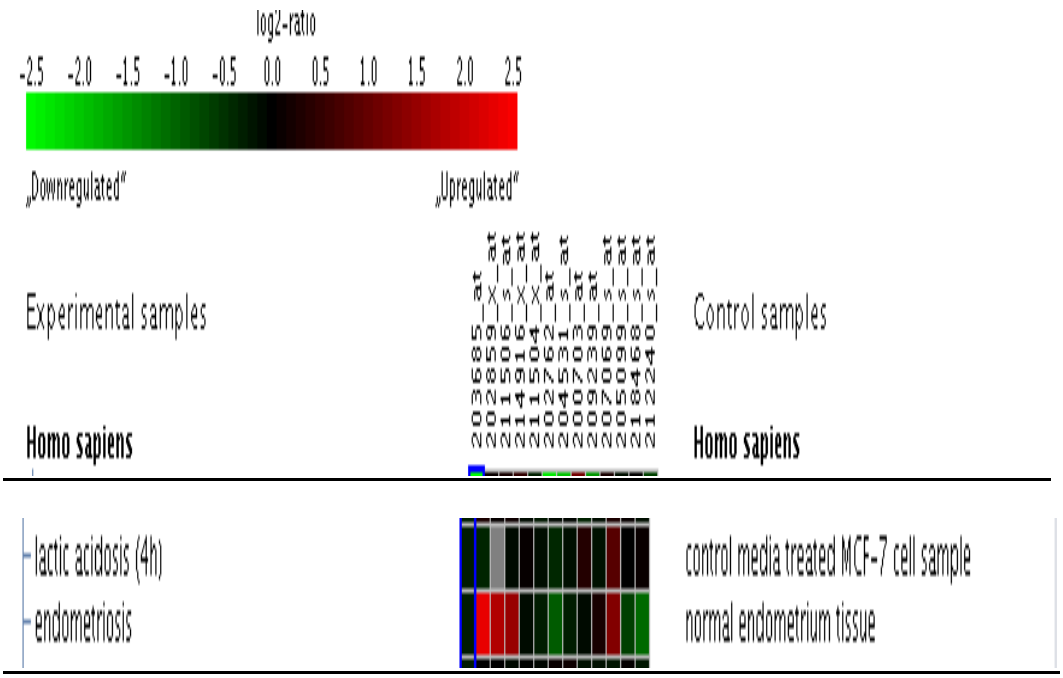
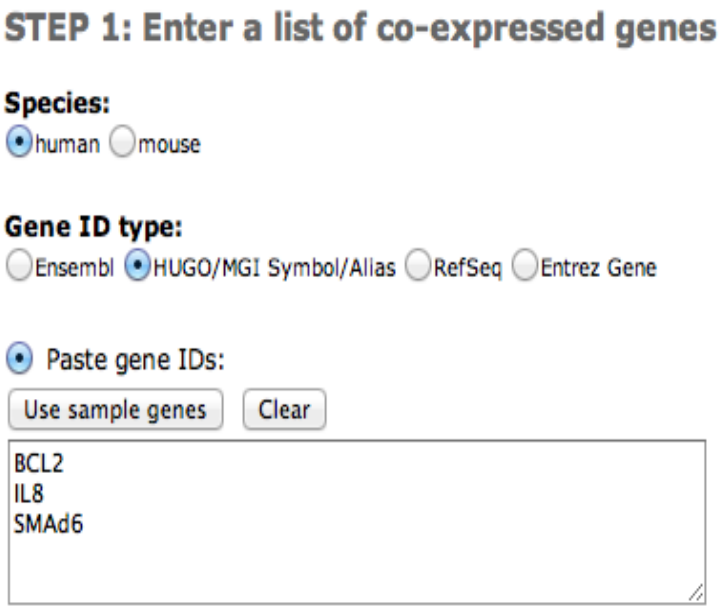


FIGURE 2.3.3.1


STEP 1: Enter a list of co-expressed genes
Species:
human
mouse
Gene ID type:
Ensembl
HUGO/MGI Symbol/Alias
RefSeq
Entrez Gene
Paste gene IDs:
Use sample genes
Clear
BCL2
IL8
SMAd6

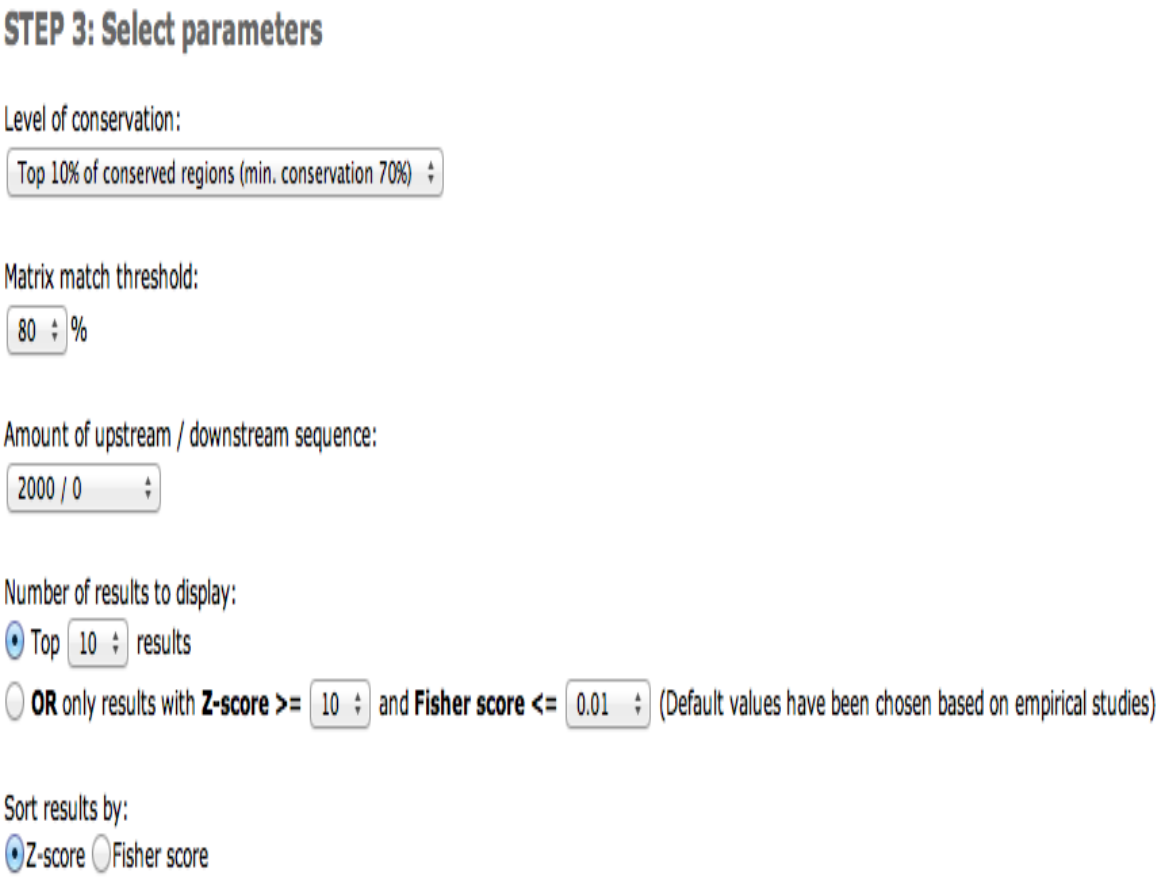
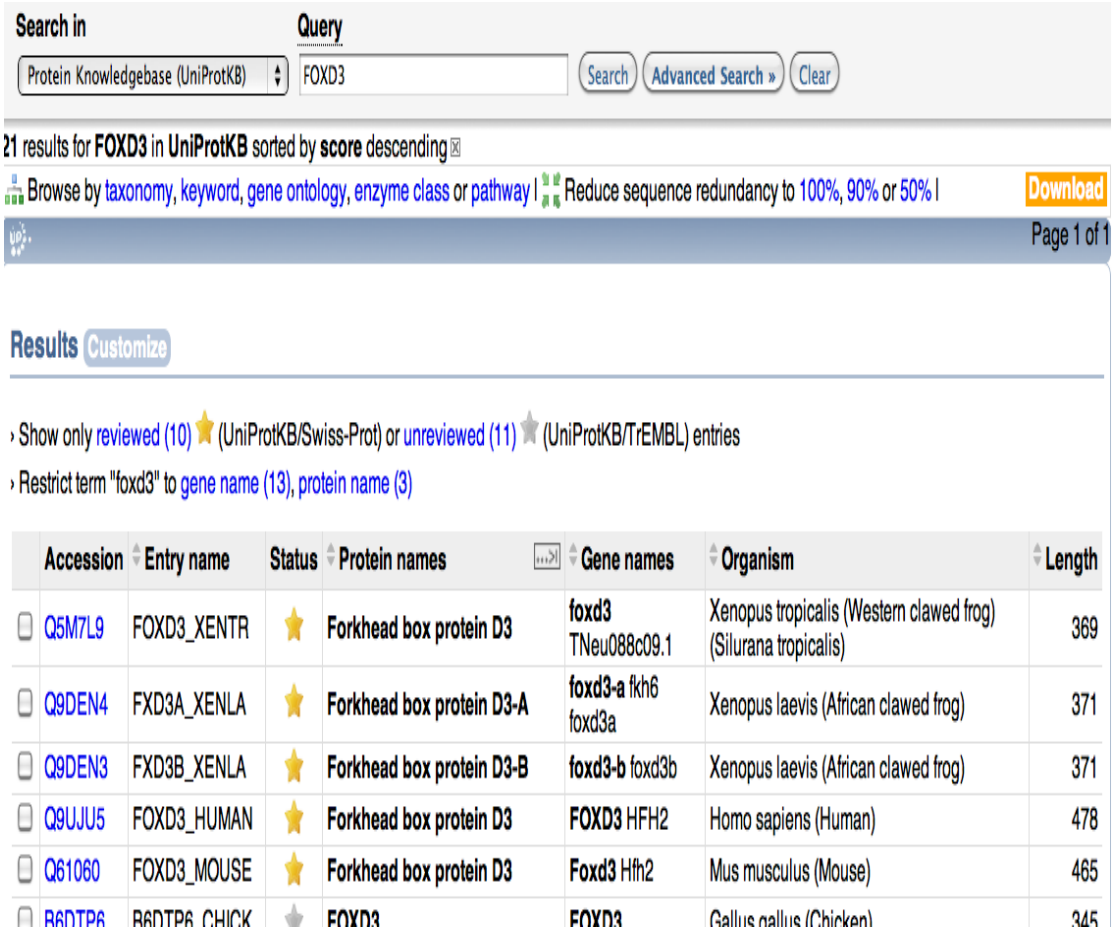

STEP 3: Select parameters
Level of conservation:
Top 10% of conserved regions (min. conservation 70%)
Matrix match threshold:
80 %
Amount of upstream / downstream sequence:
2000 / 0
Number of results to display:
Top 10 results
OR only results with Z-score >= 10 and Fisher score <= 0.01 (Default values have been chosen based on empirical studies)
Sort results by:
Z-score Fisher score

FIGURE 2.3.4.1



CHAPTER 3- DATA AND ANALYSIS

3.1 BIOINFORMATICS DATA

STEP 1

Selection of Raw Data inputted into Ingenuity IPA.

Table 3.1- A LIST OF ALL GENES/PROTEINS/MOLECULES RELATED TO ENDOMETRIOSIS INPUTTED INTO INGENUITY

MAOB	Bcl2	BTRC	CFL1	WT1	RB1	SP1	IDO1	MMP2	Q6PFW1
MEG3	bcl2l13	C3	CFL1	P19544	RGS12	Sp110	IFNA1	MMP3	O43314
MIF	BIRC3	c7orf23	Cks1	Q06250	RhoA	SPARC	IFNAR1	MMP9	PRDX6
MIR21	BMI1	CBARA1	CLIC1	Q9ULE0	RNH1	spp1	IGF1	MRC1	PRDX6
mki67	BMP7	CCL1	COMT	Q9GZV5	SOX15	ERBB4	IGFBP6	MUC16	PROK1
MME	Dak	CCL2	CRH	Q6GPH4	SOX2	F2R	IHH	muc3	PSAP
VCAN	DEFA1	CCL20	CRHr1	P98170	DICER1	FAM38A	IL15	MYH7B	PTEN
P18206	DEFB125	CCL20	CSN3	XIAP	DIO2	FASLG	IL17A	NANOG	PTGER3
Q96JH7	DFFA	CCL5	Cthrc1	TGFB1I1	DKK1	FCGR3A	IL18	NCAM1	PTGES
VEGFA	Q9BT76	CCL5	CXCL1	TGFBR2	DNMT1	FGF9	IL1	NFKB1	PTGS2
VEGFC	Q8TCY9	CCNE	CXCL14	TNFa	DNMT3a	VEGFD	IL1R1	C7orf3	PTPN22
Q96I51	O75445	CCNE2	CXCL14	Tnfrsf1a	DNMT3b	fkbp4	IL8	NOS3	Ptpn22
Acta2	O60763	CCR1	CXCR4	SERPINA3	DPPA2	Q6ZQN3	JUN	NOX1	A8MTW9
ADPRHL1	UTF1	CCR6	CYP19A1	SF1	DUSP1	FOXO1A	KCNK2	nPR2	LTF
AGR2	UTF1	CD163	CYP1B1	SFRP4	E2F1	FRAT1	Kcnma1	NR2F1	MAOA
AhR	Q9UBK9	CD36	CYP26A1	SFTPA1	EGFR	GDF3	Q69YN4	ntrk2	ROCK1
AKR1B1	P23763	CD44	CYR61	Skp2	Egfr	GRAP	KIR2DL2	otx1	S100A1
AKT1	P51809	CD55	PSCDBP	Q05940	EGFR	H2AX	KIR2DS5	OT	S100A4
AW320017	Q99536	Cdh1	O75717	STAT	EHD2	HNF1b	KIR2DS4	OXTR	S100A8
ARHGAP20	Q9UIW0	cdk5	O75083	SOD2	eif3a	HOXA10	GPR54	PAPPA	S100P
Arnt	TAGLN	CDK6	Q64LD2	SOX1	ELANE	HOXA11	KLF4	PBK	SALL4
ATM	TCF7L2	CDKN2BAS	Q9H7D7	THEG	ERK	HRAS	KLF9	PDCD4	SALL4
ATP2A2	TCL1a	TERT	P57081	TWIST1	ERAS	HSD17B2	LAMA5	PGD	SERBP1
ATP2C1	TGM2	TGFb	P61964	TXNIP	ERBB2	hspa1a	LAMB1	BB114106	
Bax	TLR2	ubl3	Q6PJI9	StAR	ERBB3	HTRA1	Q86TA4	pls3	

TABLE 3.2: A LIST OF GENES/MOLECULES/PROTEINS RELATED TO PROLIFERATION IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

Raf-1	7TM	HB24
B-raf	c-Fos	CD147
ROCK-II	fkbp52	GREM-1
sst1	cdc2	LXA4
sst2	IL-8	BLT1
NFKB1	FAK	BLT2
PELP1	ARNT	MCP-1
SICA2	TDGF1	PAR1
CCL14	SPINT1	PPACK
ALDH	FoxM1B	CYP19
ALDH10	FOLR1	p27kip1
VEGF-A	GPR30	RASK
IGF-1	NR5A1	bcl-2
		DLX4

TABLE 3.3- A LIST OF GENES/MOLECULES/PROTEINS RELATED SENESCENCE IN ENDOMETRIOSIS INPUTTED IN INGENUITY

ARNT	CD36	flt-1	GSPT1	FBLN1
AhR	MSH2	KDR	NKR1	DLX5
HSD17B1	Hic-5	AhRR	G1057D	HSD11B2
TNFR2	RCAS1	KIR2DS5	hMSH2	RHOE
TNFA	CYP19	SOX-2	SLPI	p21waf1
hMLH1	CYP1A1	stmn1-b	ENDO-1	MMP1
PTEN	TIMP-2	IL-18	SHBG	MMP3
Pal-1	CYR61	CXCR8	AR	gas6
plk1	EST	CFL1	TRKB	IL-4
MMAC1	STS	CDKN2BAS	CD94	IAP
NKG2A	JAZF1	P450Arom	NKG2A	GSTT1
IGFBP5	KRAS	NFKB1	WTN7A	GALT
PIM2	TNPR	TBP-2	EMX2	APOA2
RPL41	GSTM1	IL-6	CCL21	p16Ink4

TABLE 3.4- A LIST OF GENES/MOLECULES/PROTEIN RELATED TO IMPLANTATION IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

alox1	EGF	HTRA1	PROK1	STAT5
APOA1	FOXO1	LOXL1	SERPINA3	
BMP2	GDA	MUC1	SFRP4	
CD44	HOXA10	NR2F2	SPARC	
CD74	HOXA11	PAPPA	OPN	

TABLE 3.5- A LIST OF GENES/MOLECULES/PROTEINS RELATED TO IMPLANTATION IN THE ENDOMETRIUM INPUTTED INTO INGENUITY

Bmp2	Dll4	HOXA10
BMP7	EMP2	HPSE2
CD44	FGF7	IGF2

CD52	foxa2	IGFBP1
CFLAR	FUT7	IGFBP3
CHMP1A	GLI1	IHH
CSF2	GLI2	IL11
CXCL1	GPR30	IRF1
cyp26a1	GPER	iTGA3
DEDD	H19	MMP3
DKK1	HIF1A	MUC1
Dkk2	SPP1	NDRG1
PTCH1	STAT3	PCK2
RXFP1	STC1	PACE4
RXFP2	TEAD4	PRL
S100A13	Tead4	PROK1
S100A4	TM4SF4	LEF1
ITGA5	TRPV6	LOXL1
ITGB3	MET	SFRP1
ITGB4	MMP10	sFRP4
BTEB1	MMP2	GLUT1
LAMA1	s100G	LAMB3

TABLE 3.6- A LIST OF GENES/MOLECULES/PROTEIN RELATED TO OESTROGEN RESPONSIVENESS AND ENDOMETRIOSIS INPUTTED INTO INGENUITY

BMP2	CYP19	ESR2	IL1R2
CCL11	CYR61	FHL2	IL1R2
CCR9	CYR61	GSTM1	JUND
cdc2	DKK1	HSD17B1	MMP3
CXCL12	ESR1	HSD17B2	STAT5
CXCR4	WT1	HSD17B2	PTEN
CYP19A1	SF1	HSD3B1	STS

TABLE 3.7-A LIST OF GENES/MOLECULES /PROTEINS RELATED TO INFLAMMATION IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

BRCA1	COL5A2	HLA-G	IL8
BRCA2	GRO1	HPGD	PLAT
CCL21	GRO2	IL10	PPARG
COL4A2	GnRH	IL6	S100A8

TABLE 3.8- A LIST OF GENES/MOLECULES/PROTEINS RELATED TO RETROGRADE MENSTRUATION IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

ACE	CXCL12	MMP1	MMP9

BSG	CXCR4	MMP2	PRDX2
c17orf63	GALT	mmp24	PAI1
CFL1	GAPDH	MMP3	TIMP2
CHRNB1	HSPB1	MMP7	Twsg1
XRCC1			

TABLE 3.9- A LIST OF GENES/MOLECULES RELATED TO METASTASIS IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

NOD2	bcl2	GREM1
PAX8	COL6A1	GREM1
SDC2	COL6A2	IL1
THBS1	Erg	IL6
VEGFA	ETS1	IL8
HOX10	Flt1	LAMA4
WNT4	GnRH	MMP1
WNT5A	MMP2	WNT7A

TABLE 3.10- A LIST OF GENES/MOLECULES ETC RELATED TO CALCIUM SIGNALLING IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

AKT1	CYP19A1	HSD17B2	PTEN	spp1
Arnt	CYP1B1	KCNK2	PTGS2	SQSTM1
ATM	eif3a	Kcnma1	S100A4	StAR
ATP2A2	FGF9	mki67	S100P	STAR
ATP2C1	H2AX	NANOG	SALL4	TCL1a
BMI1	TCL1a	NR2F1	SF1	TERT
spp1	TERT	WT1	SOX15	TNFa
SQSTM1	TNFa	ZFP42	Q96RL1	UTF1
StAR				

TABLE 3.11- A LIST OF GENES/MOLECULES RELATED TO PROSTAGLANDINS IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

Acta2	DIO2	ERBB4	IGFBP6	IL1R1
BMP7	DKK1	VEGFD	IHH	ntrk2
CCL20	DUSP1	FOXO1A	IL15	SP1
CCR6	E2F1	HTRA1	IL17A	VEGFC
CD55	EGFR	IDO1	PSAP	KLF9
CXCL14	egfr	IFNAR1	pls3	LAMB1
CYP26A1	ERBB2	IGF1	PDCD4	LTF
DICER1	ERBB3	PTEN	PAPPA	SPARC
VEGFC	VCAN	MMP2	TXNIP	MIR21

TABLE 3.12 -A LIST OF GENES/MOLECULES RELATED TO APOPTOSIS IN ENDOMETRIOSIS INPUTTED IN ENDOMETRIOSIS

AKR1B1	DFFA	Il8	XIAP
Bax	DICER1	MIR21	ZEB1
Bcl2	EGFR	NCAM1	ZEB2
bcl2l13	ERK	NFKB1	
Dak	FCGR3A	PTGES	
SERBP1	Tnfrsf1a	RB1	

TABLE 3.13- A LIST OF GENES/MOLECULES RELATED TO P450 ENZYME IN ENDOMETRIOSIS INPUTTED IN ENDOMETRIOSIS

Q6ZQN3	Q6PFW1	Sox2	Q8TCY9
Q69YN4	O43314	SPP1	Q8IZJ1
Q86TA4	O75695	TERT	O95185
A8MTW9	S100A4	Q96RL1	Q9H1C4
Nanog	S100P	Q9H3U1	P13051
PCNA	Q05940	Q6ZN44	Q9BT76

TABLE 3.14- A LIST OF GENES/MOLECULES/PROTEINS ETC RELATED TO PROGESTERONE RECEPTORS IN ENDOMETRIOSIS INPUTTED INTO INGENUITY

AKR1C1	ERBB
AKR1C2	HOXA10
AR	PGR
CYP17	Pgrmc1
CYP19	PTGES3
SRD5A1	SSH1

STEP 2- Ingenuity IPA networks and interfaces showing genes differentially expressed in endometriosis in identified pathological processes.

Below are lists of genes/ proteins etc that were differentially expressed during endometriosis under the specific search categories. These were obtained in the inter-linking network system from Ingenuity.

All Bioinformatics Images are displayed at the end of Step 2 on a separate page.

TABLE 3.15- LIST SHOWING GENES /PROTEINS SHOWN TO BE EXPRESSED DURING INFLAMMATION IN PATIENTS WITH ENDOMETRIOSIS ACCORDING TO INGENUITY

BRCA1	SL00A8	GNRH1
IL10	BRCA2	PPARC
IL8	IL6	CCL21

Image 3.16 shows a screenshot of IPA interface of a network composed by a gene set listed in the table 3.15. Genes expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.17- LIST SHOWING GENES/PROTEINS RELATED TO RETROGRADE MENSTRUATION EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

BCL2	TP53	IL8	GSTT1	ICAM1
IL6	GALT	VEGFA	RA5GRF1	TNF

Image 3.18 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.17. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.19- A LIST OF GENES/ PROTEINS ETC RELATED TO ALTERED IMMUNE RESPONSES EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

ACE	INSRR	GALT	HSPB1	MMP3
MMP2	MMP24	TIMP2	MMP9	TGFB1
CXCR4	XRCC6	BSG	MMP1	TP53

Image 3.20 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.19. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.21
LIST OF GENES/ PROTEINS RELATED TO SENESCENCE EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

AHR	AHRR	CYP19A1	GA56
KDR	NFKB1	TNF	PTEN
VIM	CCL21	GALT	CYR61
KRAS	PPARG	TNFRSF1B	AR
EMX2	GSTT1	MMP1	HSD17B1
MMP3	STS		

Image 3.22 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.21. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.23

LIST OF GENES/ PROTEINS RELATED TO ESTROGEN RESPONSIVENESS EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

CDK1	DKK1	HSD17B2	MMP3	PTEN	CXCR4	ESR1
HSD17B4	MUC1	STS	MARCK5	OVGP1	CYP19A1	ESR2
IL1R2	NFKB1	WT1	CYR61	HSD17B1		

Image 3.24 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.23. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes/proteins in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.25-
LIST OF GENES/ PROTEINS RELATED TO METASTASIS EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

BCL2	FLT1	IL8
COL6A2	IL6	SDC2
IL1A	MMP2	CDL6A1
MMP1	XCL1	LAMA4
VEGFA	CCL28	TNF
CCL23	GNRH1	

Image 3.26 shows screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.25. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.27

LIST OF GENES/ PROTEINS RELATED TO CALCIUM SIGNALLING EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

AHR	CDK1	HSD17B2	PTGS2
SPP1	TNF	ATP2B2	CYP19A1
PTEN	RBP4	STAR	WT1

Image 3.28 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.27. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.29
A LIST OF GENES/ PROTEINS RELATED TO PROSTAGLANDINS EXPRESSED IN ENDOMETRIOSIS ACCORDING TO INGENUITY

ACTA2	TP53	LIF	EGFR	IL1R1
DKK1	NFRK2	PGR	IGFB1	DARC
FOXO1	CYP26A1	CXCR6	MMP2	EFBB2
MAOA	PTEN	DUSP1	IHH	VEGFC

Image 3.30 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.29. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (yellow circle). Genes in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.31
A LIST OF GENES/PROTEINS RELATED TO PROLIFERATION IN THE ENDOMETRIUM EXPRESSED IN THE ENDOMETRIOSIS ACCORDING TO INGENUITY

BCL2	CYP191A	IL8	ROCK2	BRCA1	CDK1	DYNLL1
SMAD6	CCR1	FPR2	NRSA1	GREM1	PIK3R1	NFKB1

Image 3.32 shows a screenshot of the IPA interface demonstrating a network composed by a gene set listed in the table 3.31. Genes from this table expressed during endometriosis are shown by links to 'endometriosis' label (red circle). Genes/ in the grey boxes are those from our inputted list, whilst those in the white boxes have been formulated by the Ingenuity interface to be involved in the interlinking network.

TABLE 3.33
A LIST OF GENES/PROTEINS EXPRESSED IN PATIENTS WITH ENDOMETRIOSIS BASED ON DATA MINING USING KEYWORDS 'ENDOMETRIOSIS MICROARRAY STUDIES'.

ACTA2	IL8	CD163	CCR1	MMP3	MMP9	CXCR4	NFKB1
CCL2	MMP2	CYR61	CYP26A1	NTRK2	PBR	DKK1	PGR
CYP19A1	NOS3	DNMTI38	DNMT3A	PTGER	PTGS2	DUSP1	PTPN22
DNMT1	PTEN	FOXO1	ERBB2	S100A8	SLC18A2	GOS2	SPP1
EGFR	S100A1	HSPA1A	HSD17B2	TGFB1	TGFB1	IHH	TNF
GRAF	STAR	IL1R1	IL1A	VEGFA	VEGFC	IL6	WT1
IL18	TNFRSF1A	AHR	JUN	AR	BCL2	MAOA	

Due to their being almost 16+ interlinked networks of groups of genes/molecules/proteins related to endometriosis overall, a screenshot is not possible for thesis purposes. Instead the table shows all the genes/proteins differentially expressed in endometriosis.

BIOINFORMATICS FIGURES FROM STEP 2

IMAGE 3.16

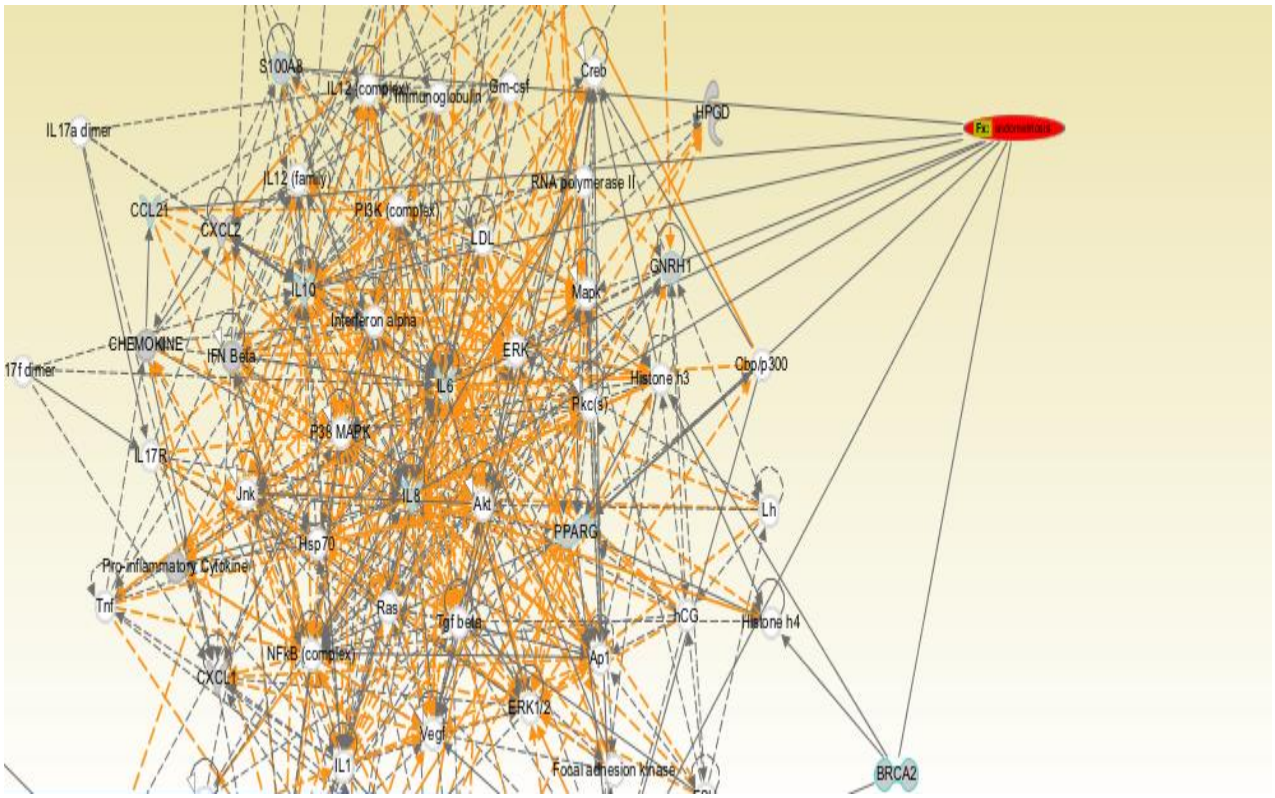
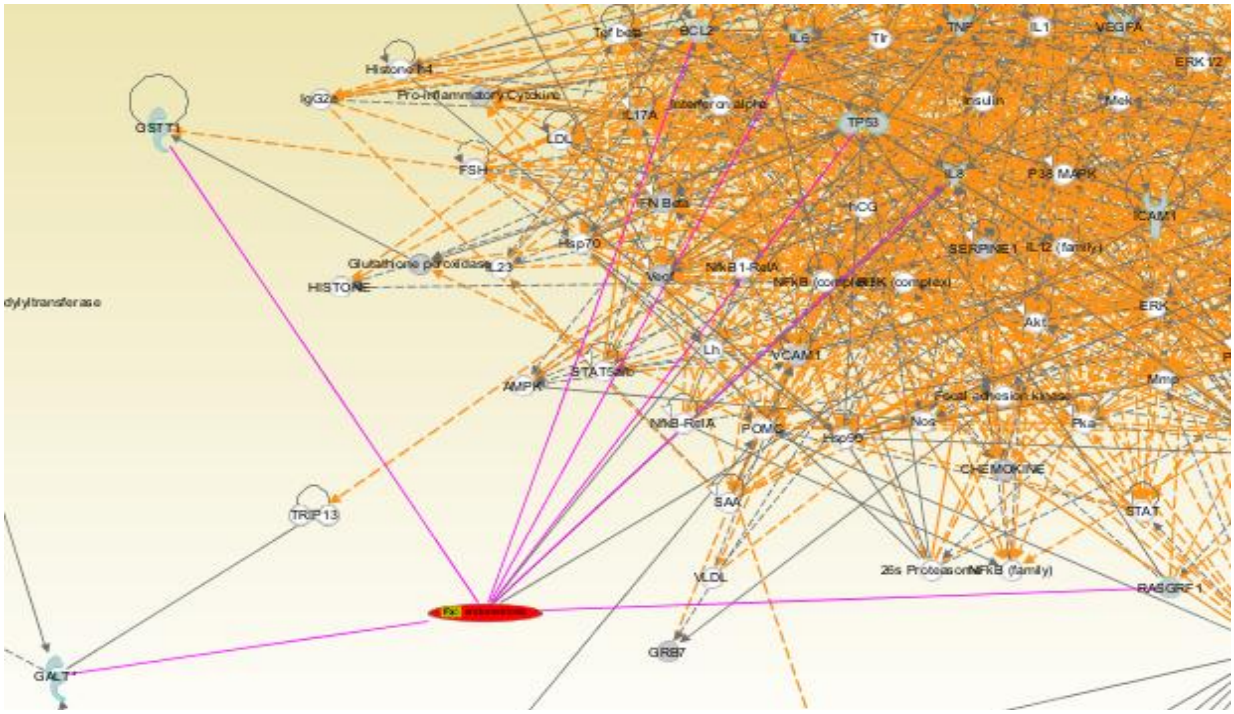


Image 3.18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Insulin
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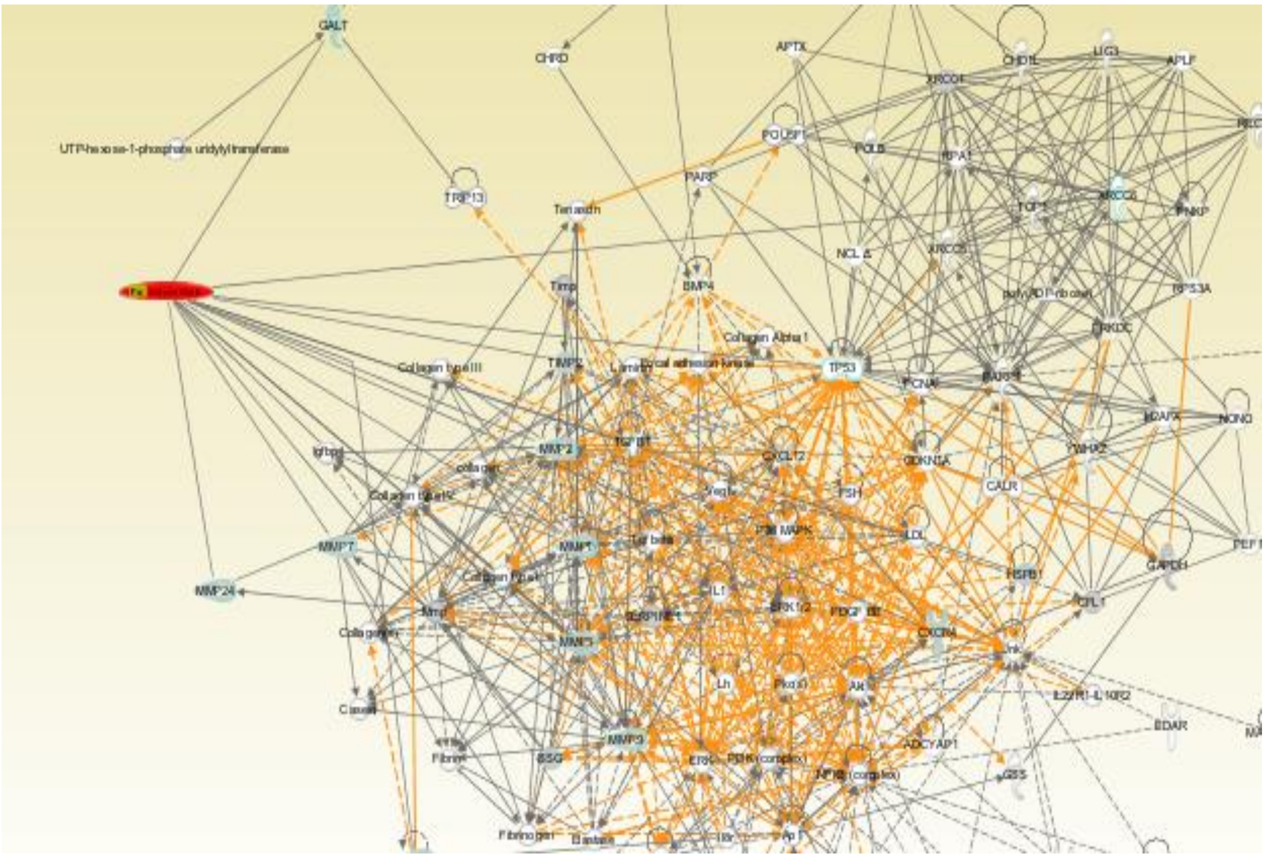
GSTT1
IL17A
TP53
LDL
FSH
IL8
P38 MAPK
IFN Beta
hCG
ICAM1
Hsp70
SERPINE1
IL12 (family)
Glutathione peroxidase
IL23
HISTONE
NfkB1-RelA
NFKB (complex)
ERK
Akt
Lh
VCAM1
STAT5alb
AMPK
Mmp
Focal adhesion kinase
NfkB-RelA
PGM3
Hsp90
Nos
Pka
CHEMOKINE
TRIP13
SAA
STAT
VLDL
26s Proteasom
NFKB (family)
RASGRF1
GRB7
GALT

IMAGE 3.20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TRIP13
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Collagen Alpha1
Focal adhesion kinase
Collagen type III
TIMP2
MMP2
MMP7
MMP24
MMP9
CXCL12
CDKN1A
CALR
FSH
LDL
P38 MAPK
HSPB1
GAPDH
H2AFX
NONO
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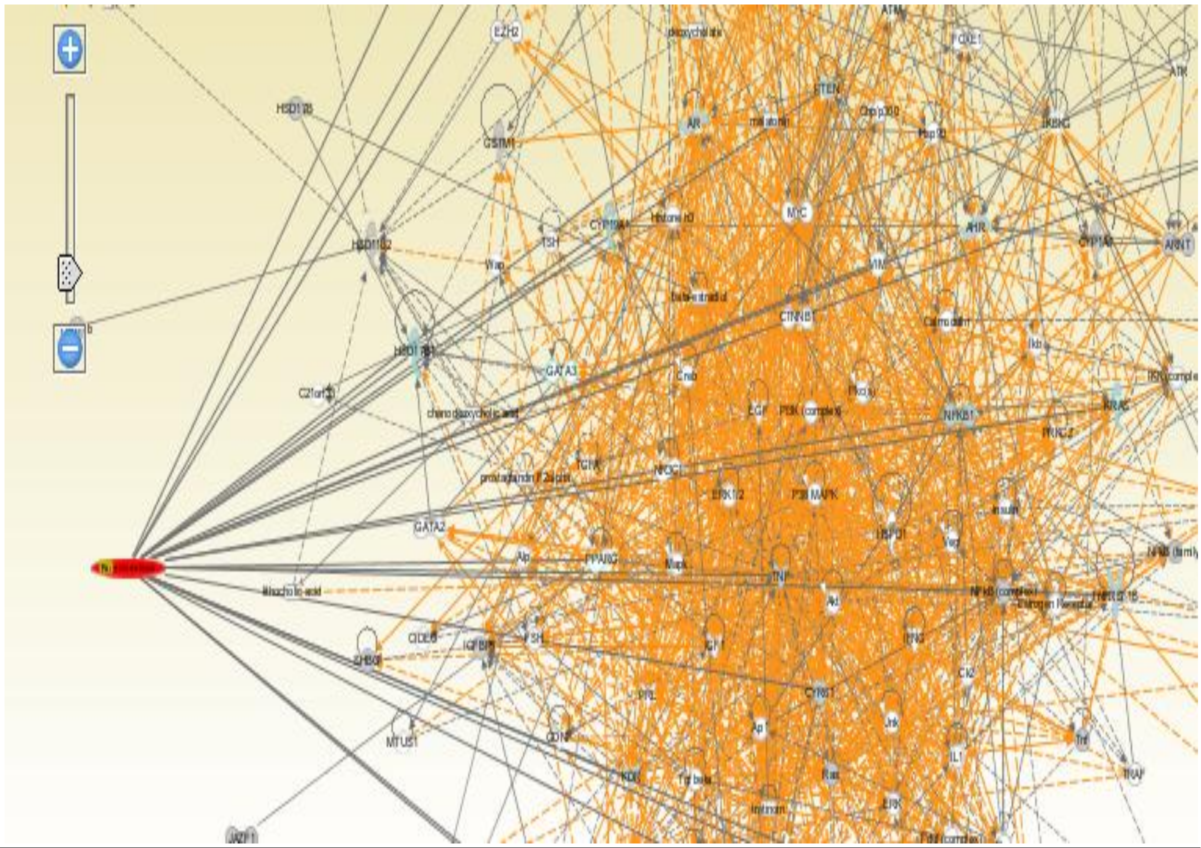
CXCR4
ADCYAP1
EDAR
PI3K (complex)
NFkB (complex)
BSG
Fibrinogen

IMAGE 3.22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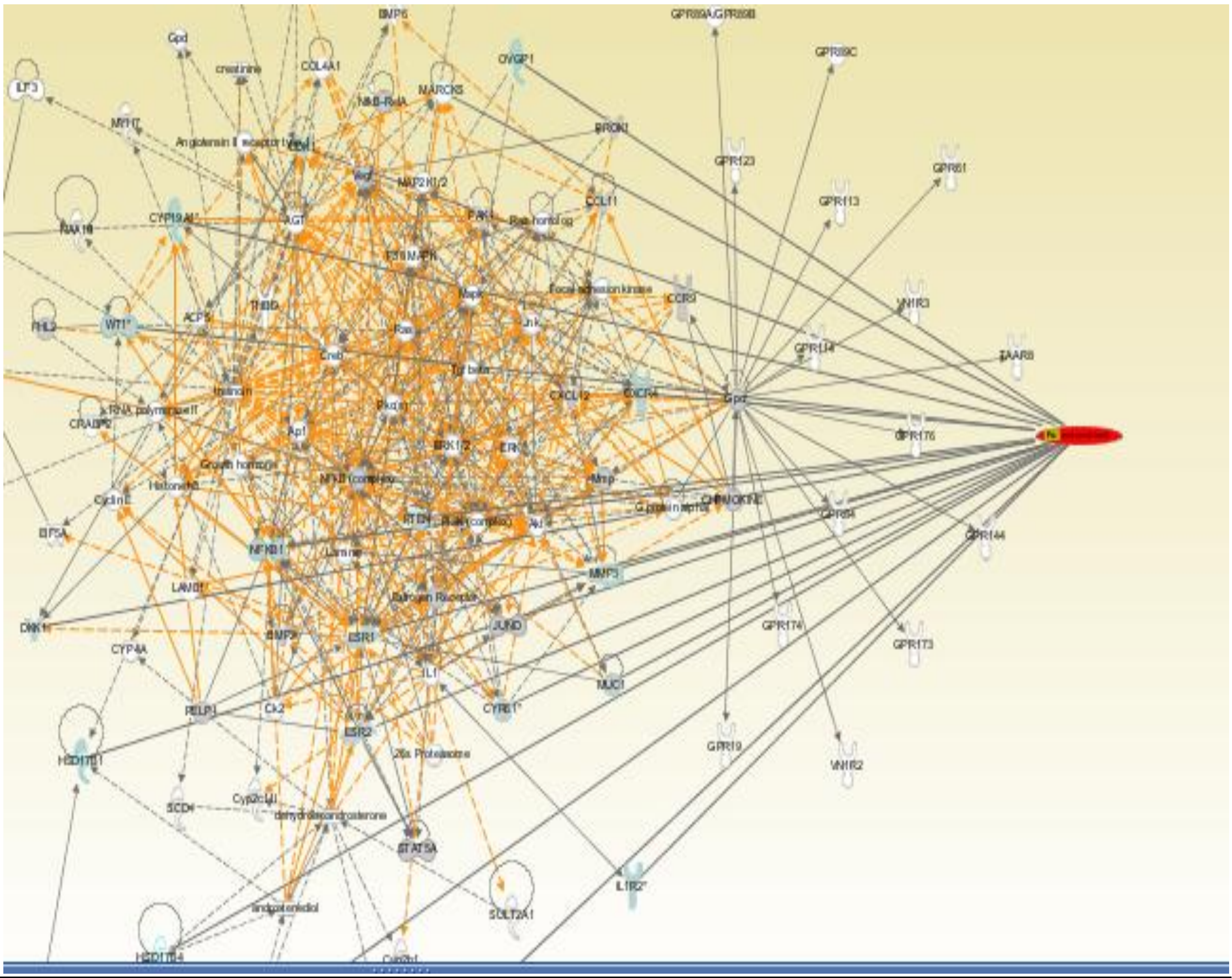
NFKB1
TNF
PPARG
PTEN
MTUS1
IL1
Insulin

Image 3.24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VN1R3
GPR114
Ras
Creb
Tgf beta
CXCL12
CXCR4
GPR176
ERK1/2
ERK
Ap1
NFKB1
Akt
GPR144
MMP3
JUND
ESR1
IL1
MUC1
GPR174
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GPR19
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STAT5A
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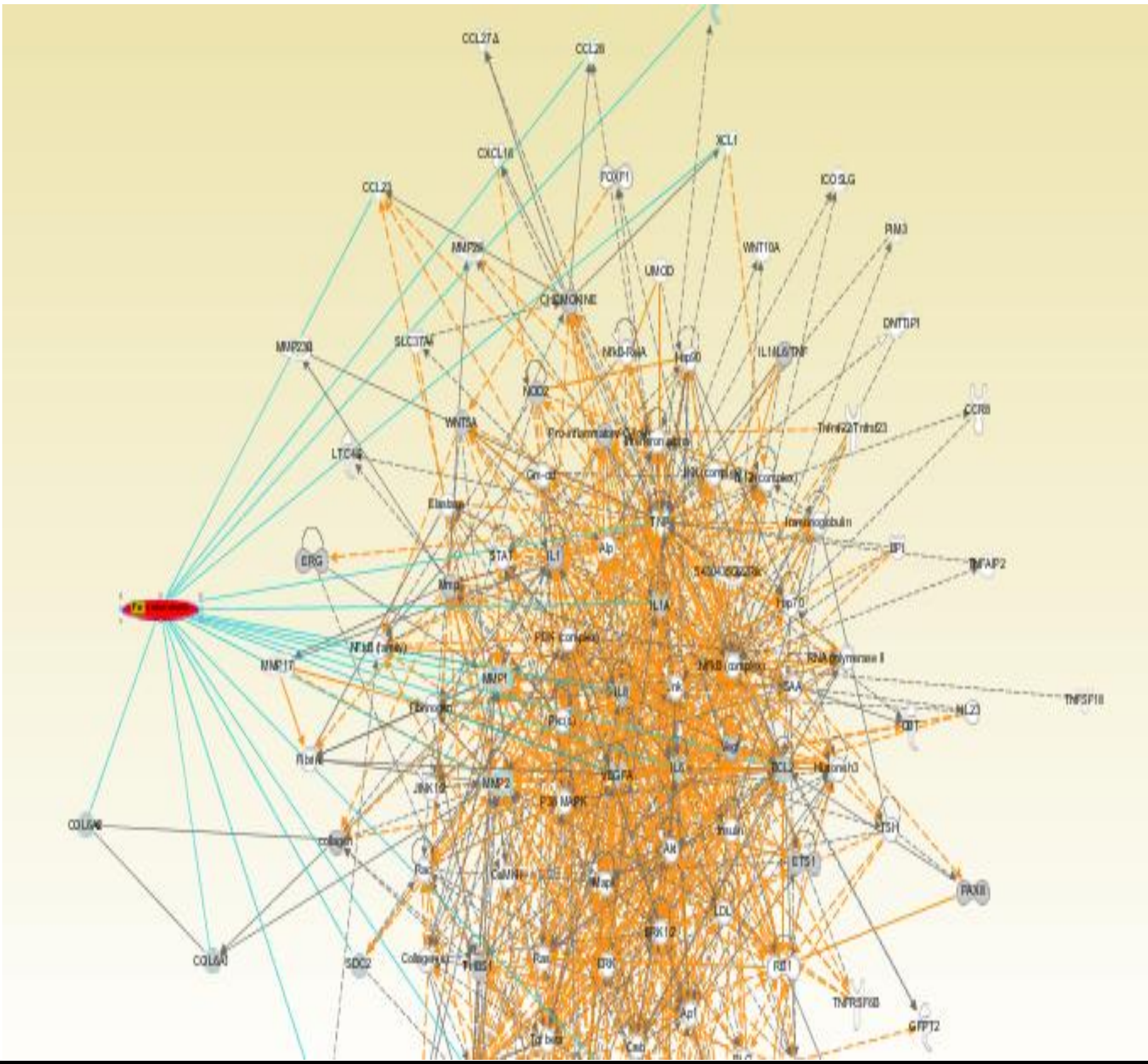
dehydroepiandrosterone

Image 3.26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LTC4S
ERG
STAT1
IL1
IL1A
MMP17
MMP1
VEGFA
P38 MAPK
JNK1/2
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IL23
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CCR8

Image 3.28

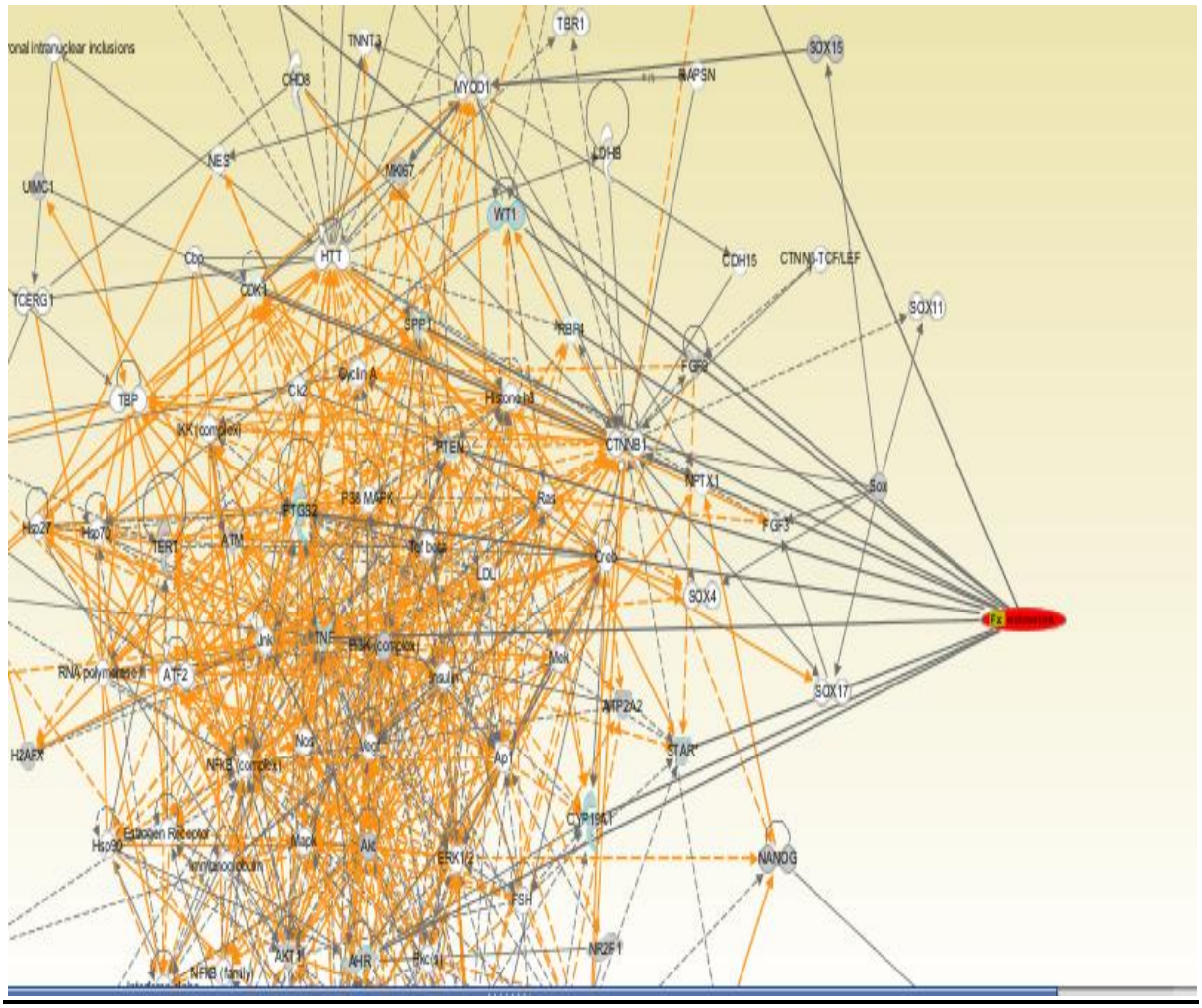
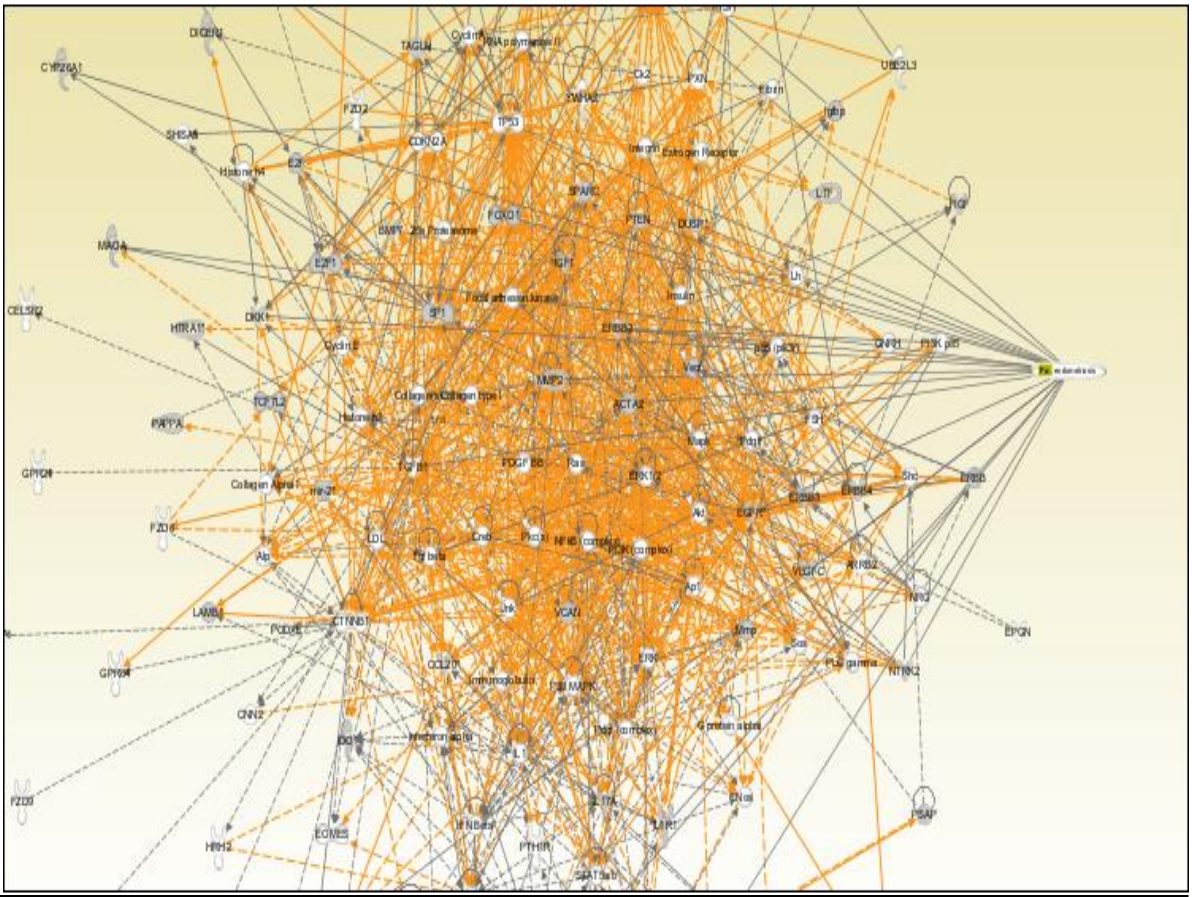


Image 3.30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SHISA5
TP53
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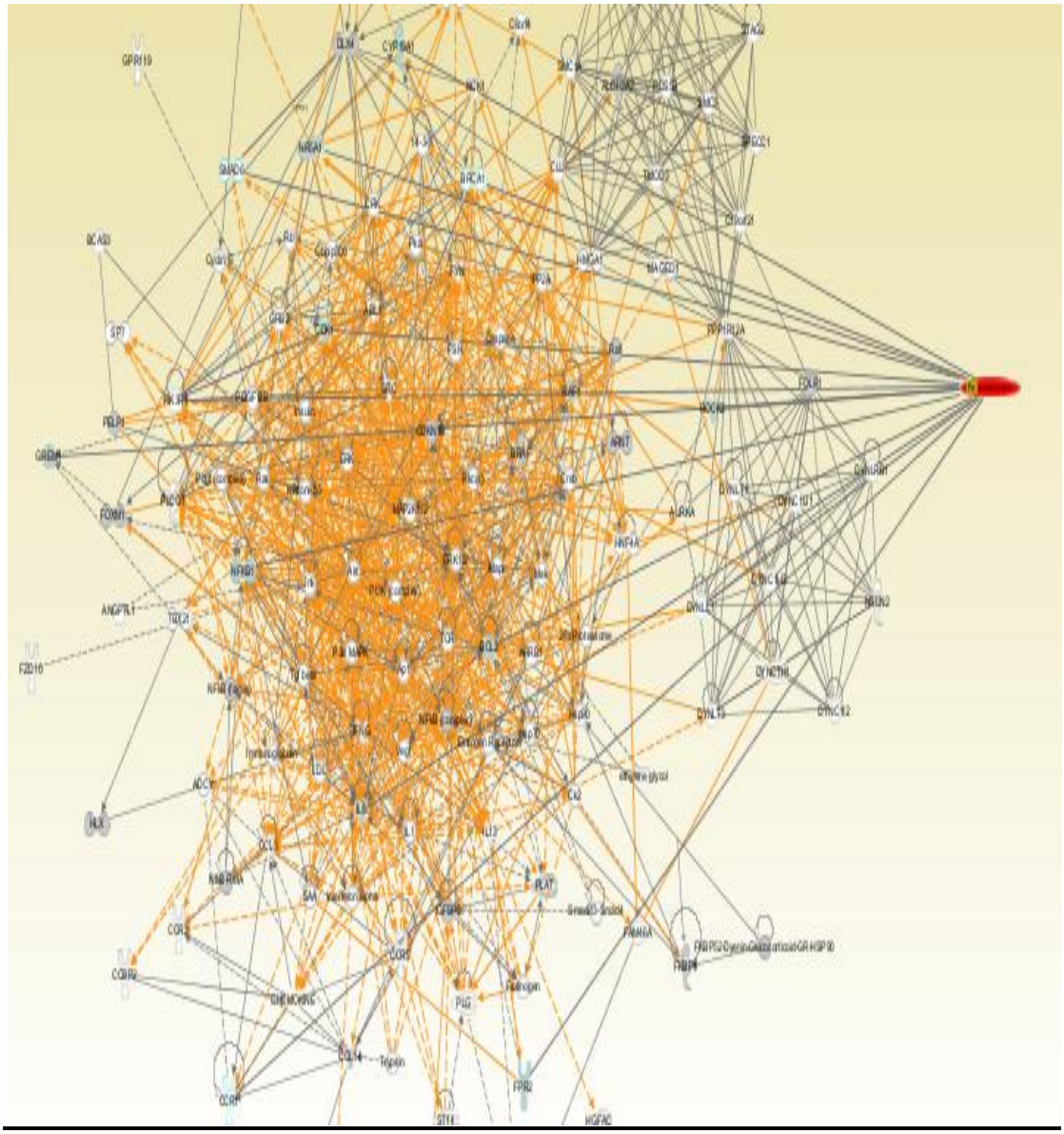
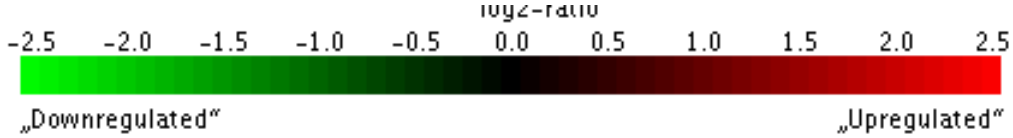
Integrin
Estrogen Receptor
SPARC
LTF
FOXO1
PTEN
DUSP1
MAGA
E2F1
Lh
Insulin
CELSR2
HTRA1
DKK1
ERBB2
GNRH
Cyclin E
MMP2
Collagen type I
ACTA2
TCF7L2
FSH
PAPPA
PDGF BB
Ras
ERK1/2
Collagen Alpha1
Shc
ERBB
ERBB4
ERBB3
Akt
EGFR
FZD6
LDL
Creb
NFkB (complex)
PI3K (complex)
VEGFC
ARRB2
Ap1
NRG
LAMB1
PODXL
CTNNB1
Jnk
VCAN
EPGN
Mmp
Sos
GPR64
CCL20
PLC gamma
NTRK2
CNN2
G protein alpha
FZD9
ENos
PSAP
HRH2
EOMES
IFN Beta
PTHR

Image 3.32



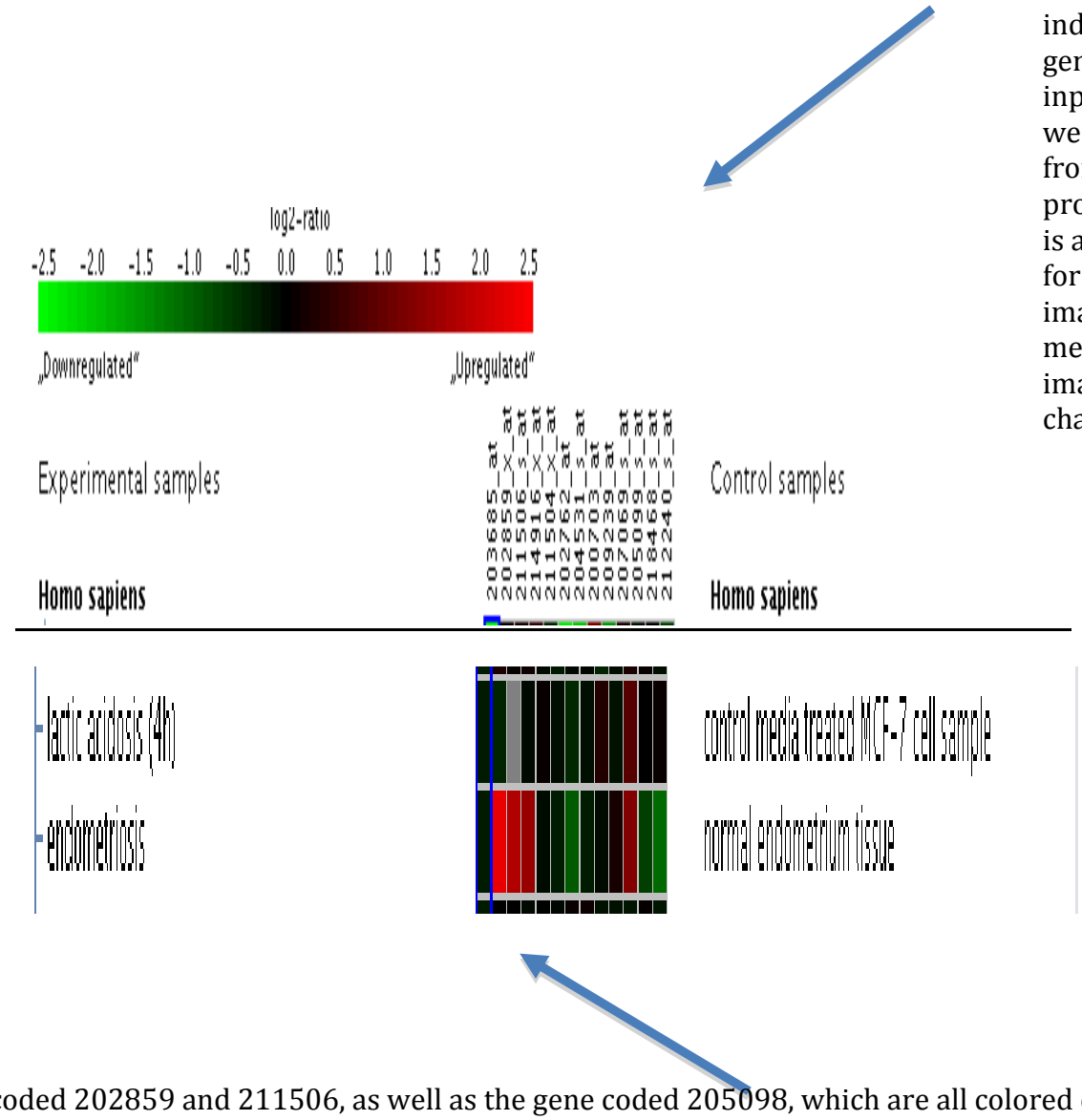
STEP 3-Discovery of genes up regulated during endometriosis through the use of Genevestigator

Our next target was to define potential regulators of differential gene expression associated with endometriosis. For this purpose we needed to know vectors of gene expression changes that were not clearly established for the majority of endometriosis associated genes. Therefore we used existing published expression data stored and validated by Genevestigator software.



The key above was used to identify which of the coded genes listed were up or down regulated. Those genes indicated in bright red were noted as being strongly up regulated whilst a majority as can be seen below, was moderately up regulated.

FIGURE 3.34
IMAGE TO SHOW A META-ANALYSIS OF THE PROLIFERATIVE GENES UP-REGULATED IN ENDOMETRIOSIS



These codes indicate the genes inputted and were decoded from the program. This is applicable for all the images of meta-analysis images in this chapter.

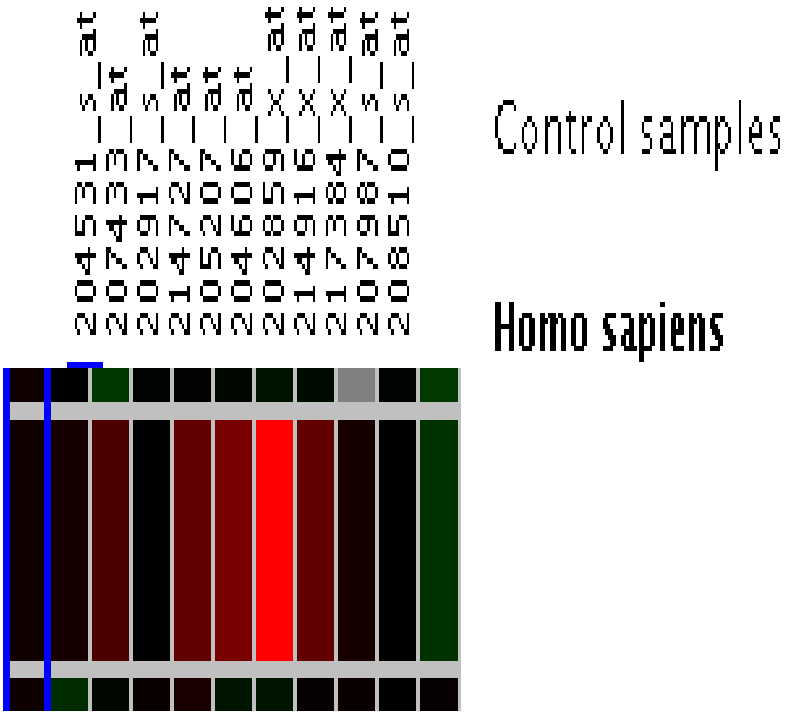
The genes coded 202859 and 211506, as well as the gene coded 205098, which are all colored deep red, are shown to be up regulated in proliferative activity during endometriosis according to meta-analysis studies. All the images (figures 3.34-3.44) below showing meta-analysis studies are arranged with the codes of genes corresponding to their level of up or down regulation listed below (either colored dark red or green).

Those up regulated were BCL2, IL8 (strongly upregulated) and SMAD6.

Listed on the left in the image showing the Meta-analysis is experimental

samples from endometriosis samples compared to control samples from normal endometrial tissue.

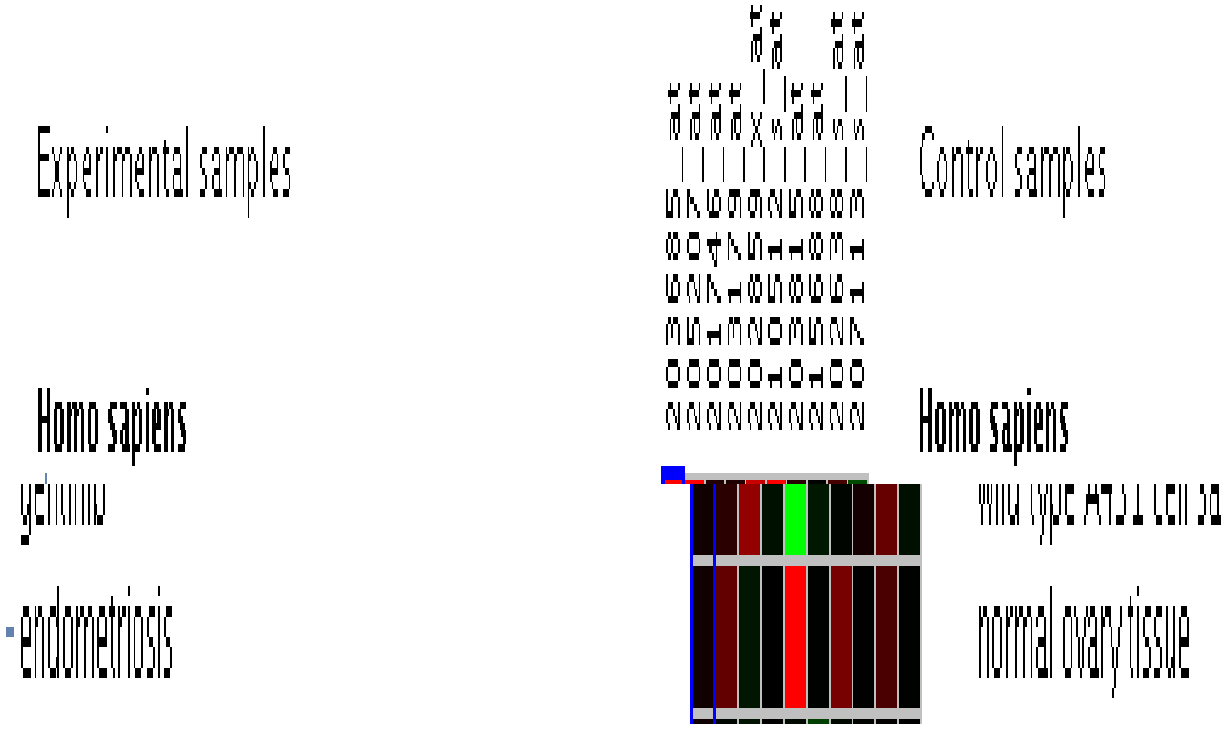
FIGURE 3.35
AN IMAGE TO SHOW THE META-ANALSIS OF GENES RELATED TO INFLAMMATION UP-REGULATED IN ENDOMETRIOSIS



Those up regulated were BRCA2, IL6, IL8 (strongly up-regulated), CCL21 and S100A8

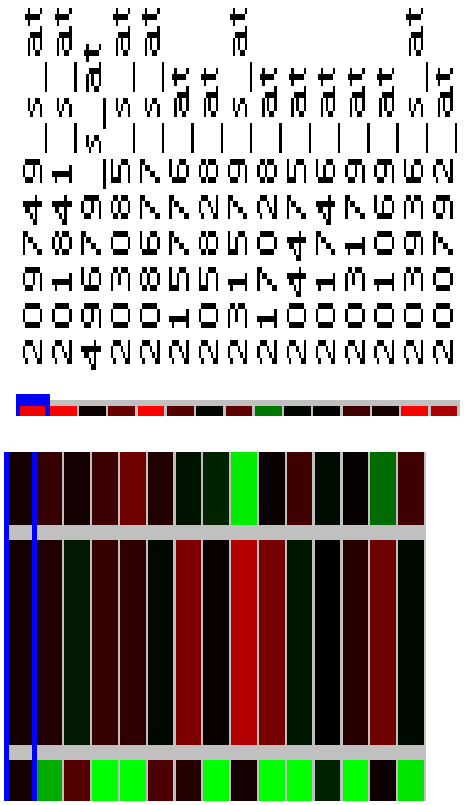
FIGURE 3.36

AN IMAGE TO SHOW THE META-ANALYSIS OF GENES RELATED TO RETROGADE MENSTRUATION UP-REGULATED IN ENDOMETRIOSIS



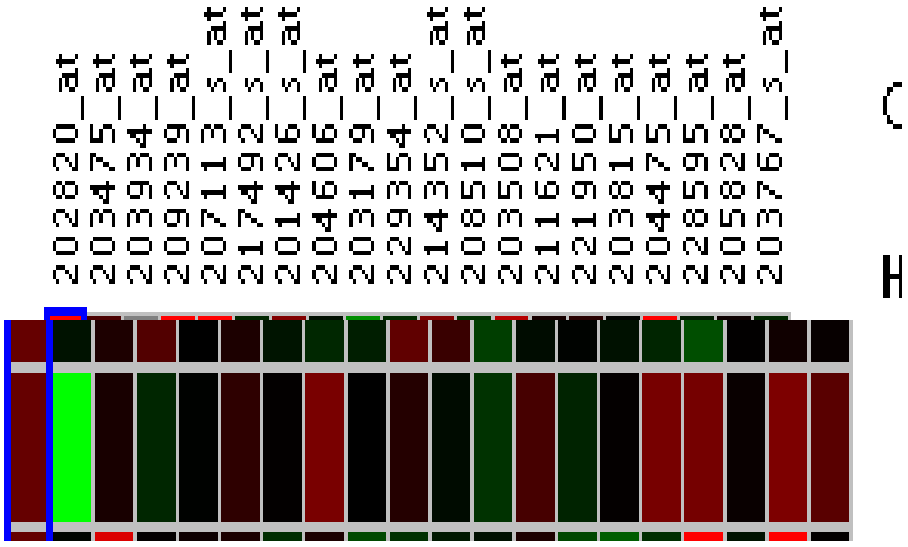
Those genes up regulated were ICAM1, GSTT, IL8 (strongly up-regulated) and IL6. Data obtained for this program was obtained in experiments using endometriosis tissues compared to normal ovary tissue as controls.

FIGURE 3.37
AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO ALTERED IMMUNE RESPONSE UP-REGULATED IN ENDOMETRIOSIS



Those genes up regulated were MMP9, MMP1, CXCR4 (strongly up-regulated), INSRR and TGFB1.

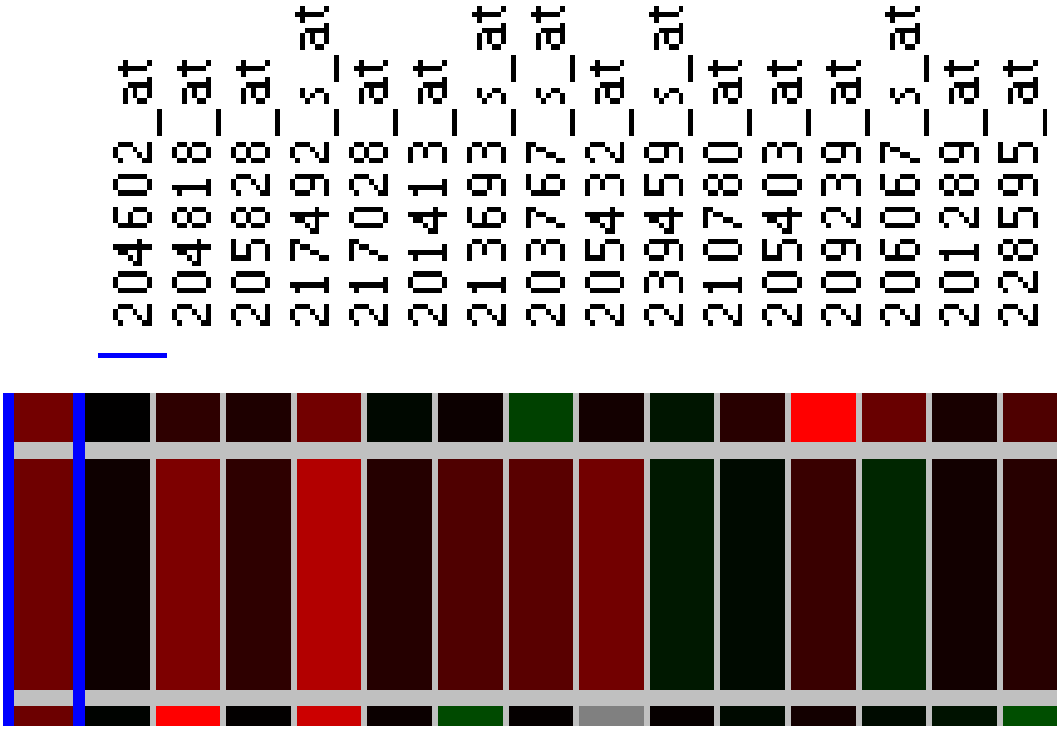
FIGURE 3.38
AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO SENESCENCE UP-REGULATED IN ENDOMETRIOSIS



Those genes up regulated were AR, NFKB1, AHR, CCL21, MMP3, MMP1 and GST11

FIGURE 3.39

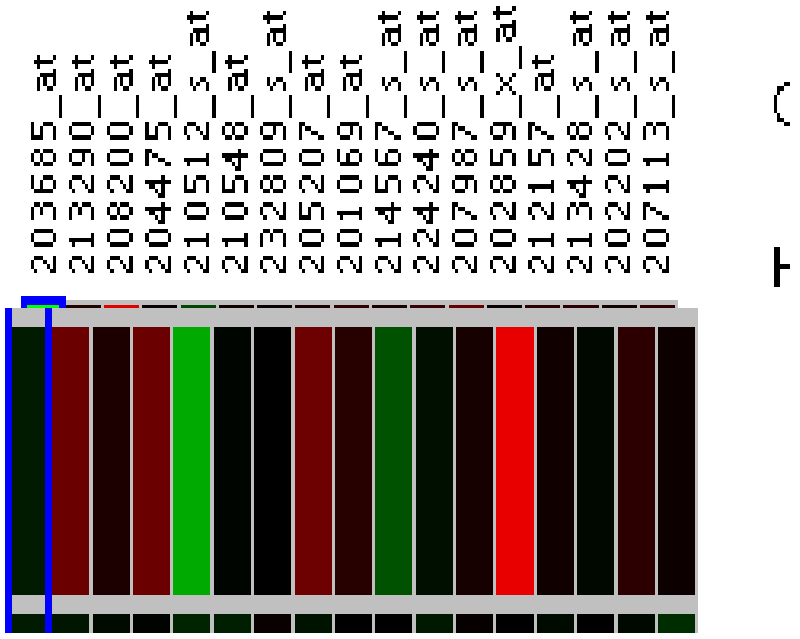
AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO OESTROGEN RESPONSIVENESS UP-REGULATED IN ENDOMETRIOSIS



Those genes up-regulated were CXCR4 (strongly up regulated) CYR61, ESR1, IL1R2, MMP3, NFKB1 and OVP1

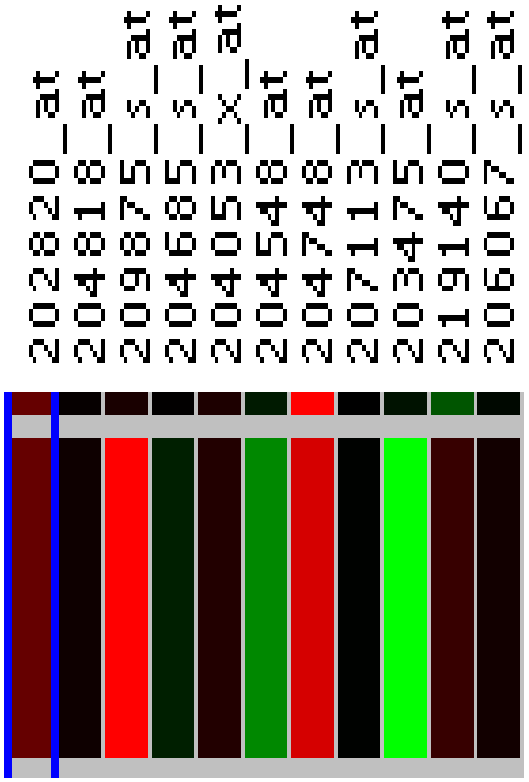
FIGURE 3.40

AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO METASTASIS UP-REGULATED IN ENDOMETRIOSIS



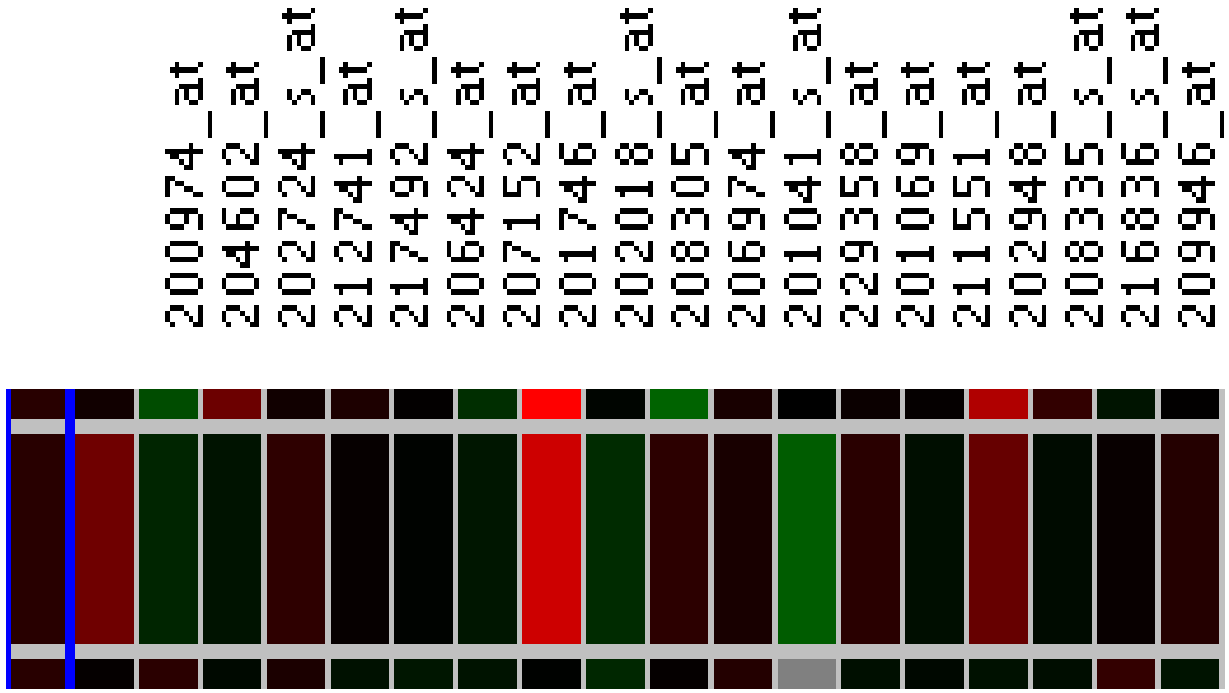
Those up regulated were LAMA4, XCL1 (very strongly up regulated), FLT1, COL6A2 and MMP1

FIGURE 3.41
AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO CALCIUM SIGNALLING UP-REGULATED IN ENDOMETRIOSIS



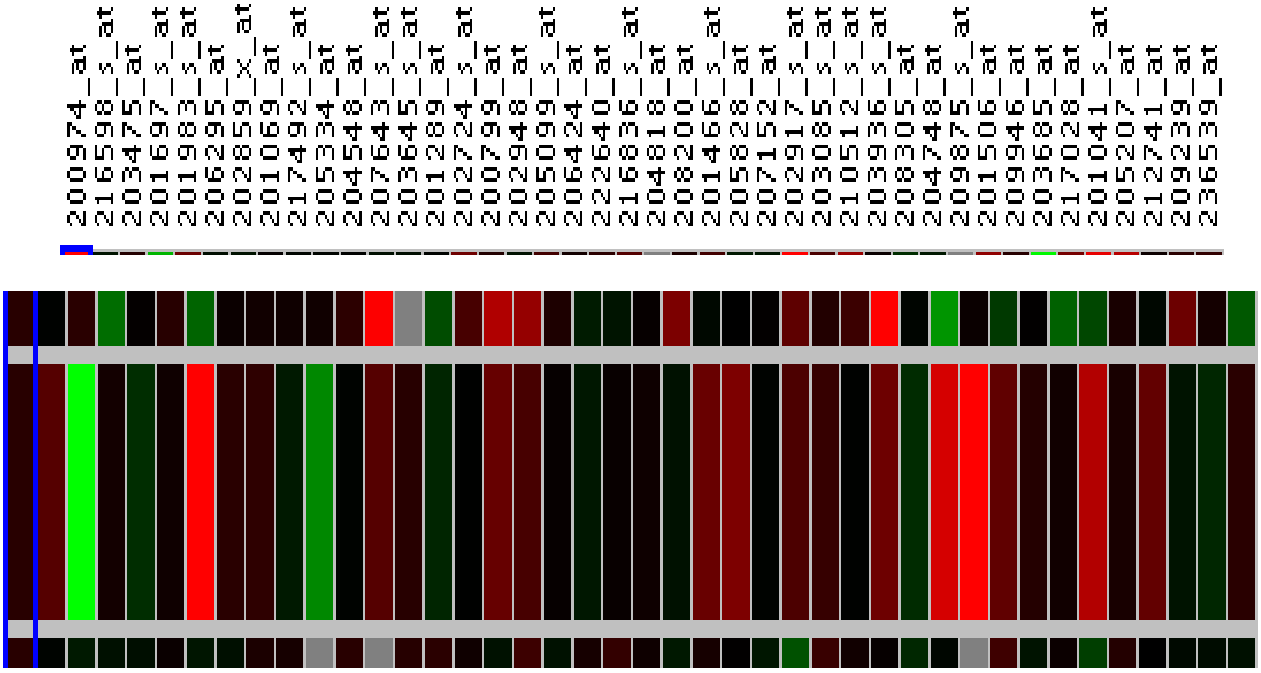
The following were up regulated AHR, SPP1 (Strongly up regulated), RBP4 and PTGS2 (Strongly up regulated).

FIGURE 3.42
AN IMAGE TO SHOW THE META ANALYSIS OF GENES RELATED TO PROSTAGLANDINS UP-REGULATED IN ENDOMETRIOSIS



Those up regulated were DKK1, IL1R2 and LTF (strongly up regulated)

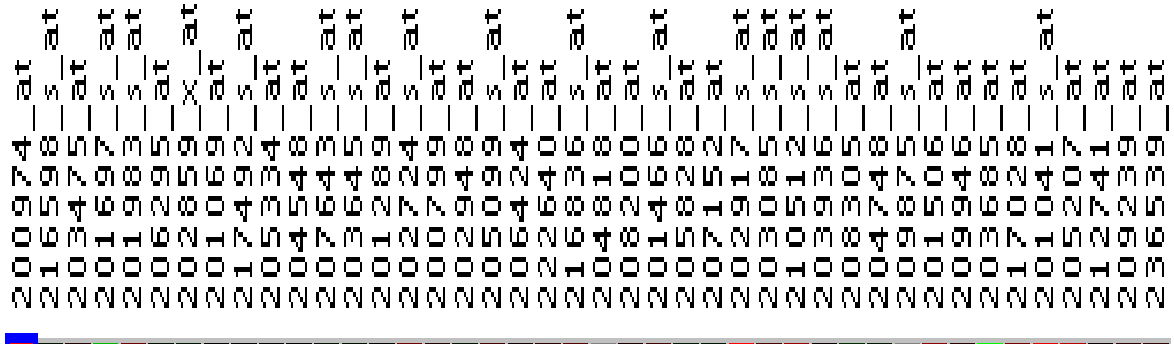
FIGURE 3.43
AN IMAGE TO SHOW THE META ANALYSIS OF GENES UP-REGULATED IN PATIENTS WITH ENDOMETRIOSIS (BASED ON FULL COMPREHENSIVE SEARCH)



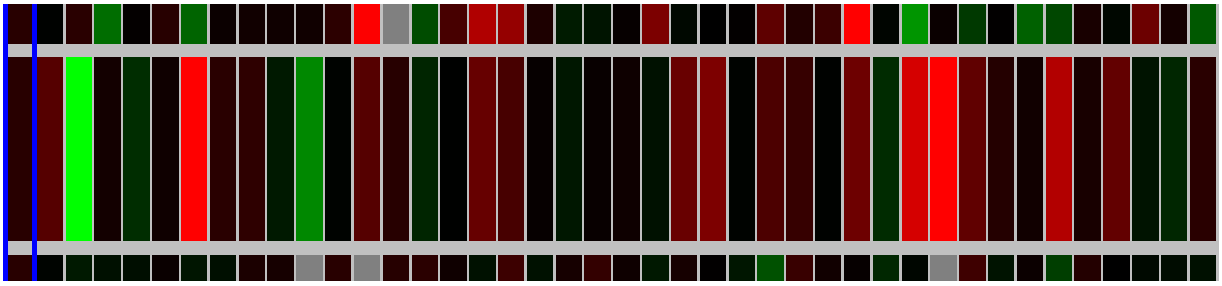
The following were up regulated:
SPP1 (Strongly up regulated)
PTGS2 (Strongly up regulated)
IL8 (Strongly up regulated)
AR
CCL2
IL6
CXCR4
SPP1
MMP9
NFKB1
PTPN22
IL1A
MMP3
STAR

FIGURE 3.44

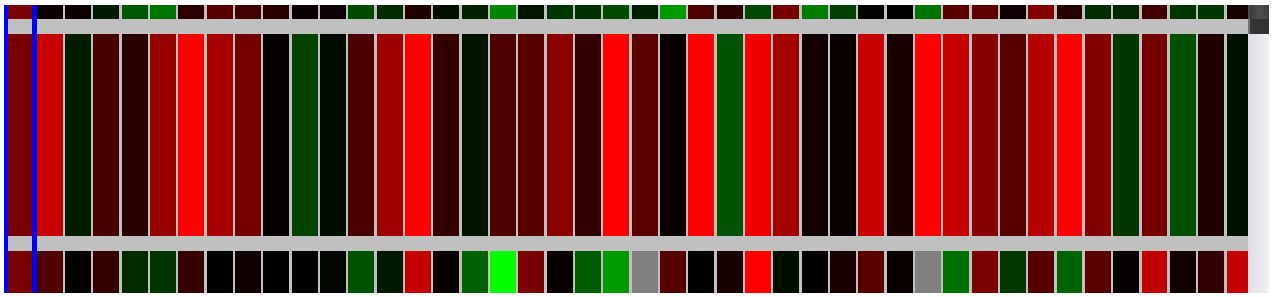
AN IMAGE TO SHOW THE COMPARISON OF META ANALYSIS OF GENES UP REGULATED IN ENDOMETRIOSIS AND THOSE REGULATED IN GASTRIC CANCER.



endometriosis



Gastric cancer



Above is a comparison of the Meta analysis studies of genes up regulated (colored in red) in both endometriosis and gastric cancer. We found the pattern of gene expression in gastric cancer from Genevistagator to be a condition that most closely resembled the expressional pattern of genes in endometriosis endometriosis. Both analysis studies showed that IL8 is strongly up regulated in both endometriosis and gastric cancer. SPP1 is up regulated strongly in both conditions, and STAR, which is strongly up regulated in gastric cancer, is also up regulated in endometriosis. CCL2 and IL6 are both moderately up regulated in both conditions.

Chapter 4: Discussion explained why some of genes/ proteins were up regulated and what their significance in the pathology of endometriosis as well as gastric cancer may be.

STEP 4- Data showing the common regulatory transcription factor for up regulated genes during endometriosis.

The next step was inputting the group of up regulated genes provided by Genevestigator into Opossum to find out which transcription factor binding sites are the most enriched in their promoter regions

TABLE 3.45
A LIST OF GENES/PROTEINS UP REGULATED IN ENDOMETRIOSIS FROM ALL SEARCH CATEGORIES

BCL2	IL8	SMAD6	SPP1	PTGS2
AR	CCL2	IL6	CXCR4	MMP9
NFKB1	PTPN22	IL1A	MMP3	STAR
CXCR4	CYR61	ESR1	IL1R2	OVP1
AHR	RBP4	DKK1	LTF	MMP1
GST11	LAMA4	XCL1	FLT1	COL6A2
CCL21	GST11	BRCA2	S100A8	INSRR
TGFB1	NFKB1	ICAM1		

TABLE 3.46
A RANKING OF TRANSCRIPTION FACTORS SHOWN TO BE CONTROLLING THE GENES UP REGULATED IN ENDOMETRIOSIS LISTED ABOVE

oPOSSUM Analysis

TF	TF Class	TF Supergroup	IC	Background gene hits	Background gene non-hits	Target gene hits	Target gene non-hits	Background TFBS hits	Background TFBS rate	Target TFBS hits	Target TFBS rate	Z-score	Fisher score
Cebpa	bZIP	vertebrate	9.187	5863	9287	17	13	12176	0.0118	39	0.0190	10.53	3.512e-02
HLF	bZIP	vertebrate	11.147	3376	11774	11	19	5014	0.0048	17	0.0083	7.744	5.289e-02
Foxd3	FORKHEAD	vertebrate	12.945	6115	9035	16	14	15856	0.0153	43	0.0210	7.197	1.047e-01
Fos	bZIP	vertebrate	10.670	7001	8149	20	10	16086	0.0104	44	0.0143	6.083	1.945e-02
IRF1	TRP-CLUSTER	vertebrate	16.008	2977	12173	8	22	4250	0.0041	13	0.0063	5.432	2.244e-01
SPIB	ETS	vertebrate	9.060	11533	3617	27	3	71785	0.0405	166	0.0472	5.379	5.000e-02
IRF2	TRP-CLUSTER	vertebrate	21.134	399	14751	2	28	424	0.0006	2	0.0015	5.244	1.874e-01
Pdx1	HOMEO	vertebrate	9.040	9899	5251	22	8	54092	0.0261	128	0.0312	4.983	2.368e-01
E2F1	E2F_TDP	vertebrate	13.838	2608	12542	7	23	3653	0.0024	12	0.0039	4.95	2.497e-01
MZF1_5-13	ZN-FINGER, C2H2	vertebrate	9.400	8290	6860	19	11	27624	0.0222	66	0.0268	4.858	2.237e-01

As can be seen, FOXD3, ranked third highest according the Z-Scoring (higher score, less of possibility scoring due to chance), was seen to be the most commonly identified transcription factor controlling some of the up regulated genes we obtained from our earlier Bio-Informatics work. It targeted 6115 out of 38 of the up-regulated genes expressed in endometriosis. It also had 43 different transcription factor binding sites.

As well as being a readily available transcription (as explained in Chapter 3:Immunohistochemistry Methodology), no work had been previously undertaken in human or animal studies investigating FOXD3 in endometriosis.

STEP 5- Data of up regulated genes with FOXD3 and its other accessions

The next stage involved using the program UniProt to find any other identifiable names for the transcription factor FOXD3 to ensure reliability when we later tried to confirm the link between FOXD3 and the group of up-regulated genes in the networking system of Ingenuity. Species were selected only from Human organisms (Homo Sapien) to provide consistency, as all of the previous bioinformatics work had been conducted using human (Homo Sapien) computational studies in all the software so far.

TABLE 3.47
A LIST OF THE GENES PROTEINS UP REGULATED IN ENDOMETRIOSIS THAT FOXD3 POTENTIALLY CONTROLS

FOXD3	INSRR	DKK1	BCL2
Q9UJU5	ESR1	SPP1	RBP4
QO1860	NFKB1	IL6	CYR61
PTGS2	LAMA4	PTPN22	AR
STAR	IL1A	SMAD1	

FOXD3 and its two other accessions are in bold
This list was inputted into Ingenuity to confirm the link between FOXD3 and the above key up regulated players.

STEP 6- Ingenuity IPA interface network confirming a link between FOXD3 and set of up regulated genes in endometriosis

We used Ingenuity to input the contents of Table 3.47 to confirm a link between FOXD3 and endometriosis.

IMAGE 3.48
IMAGE SHOWING THE RELATIONSHIP BETWEEN FOXD3 AND THE GENES UP REGULATED IN ENDOMETRIOSIS IN THE IPA INTERFACE

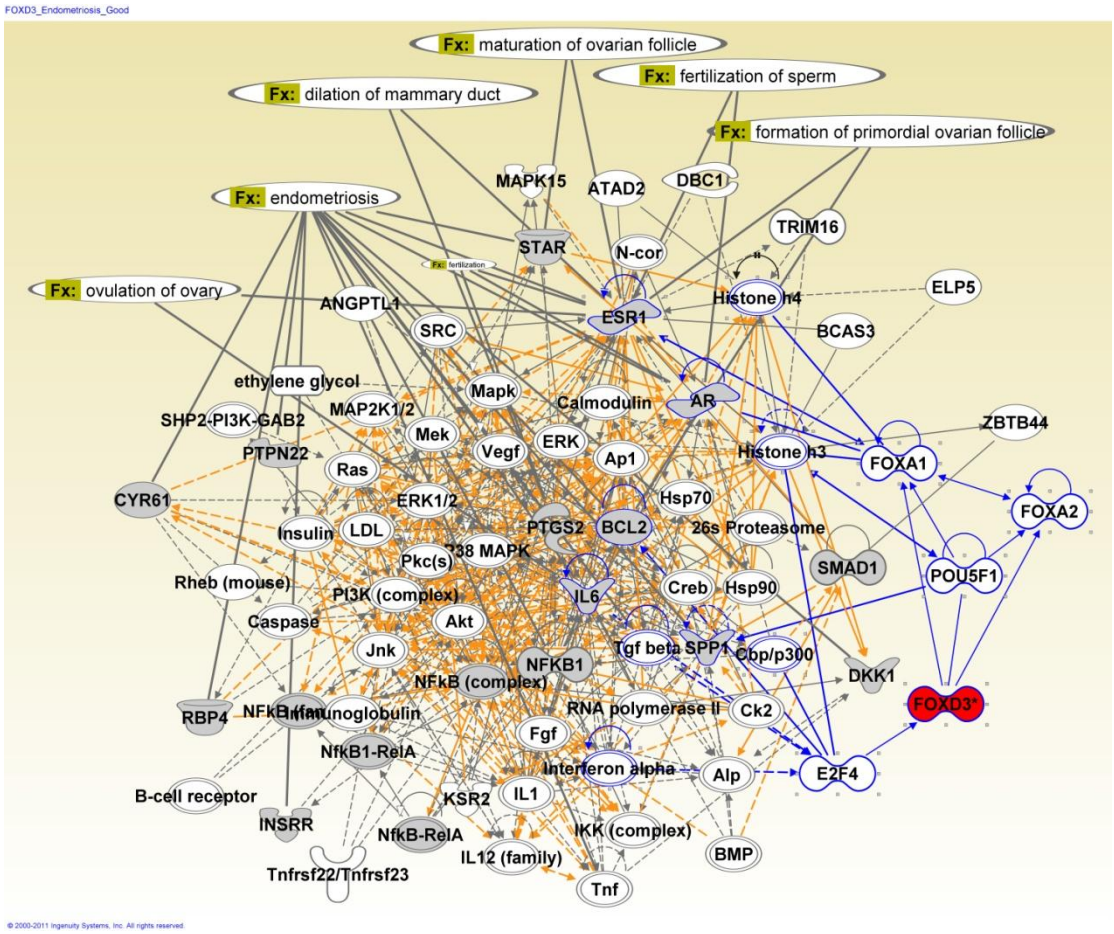
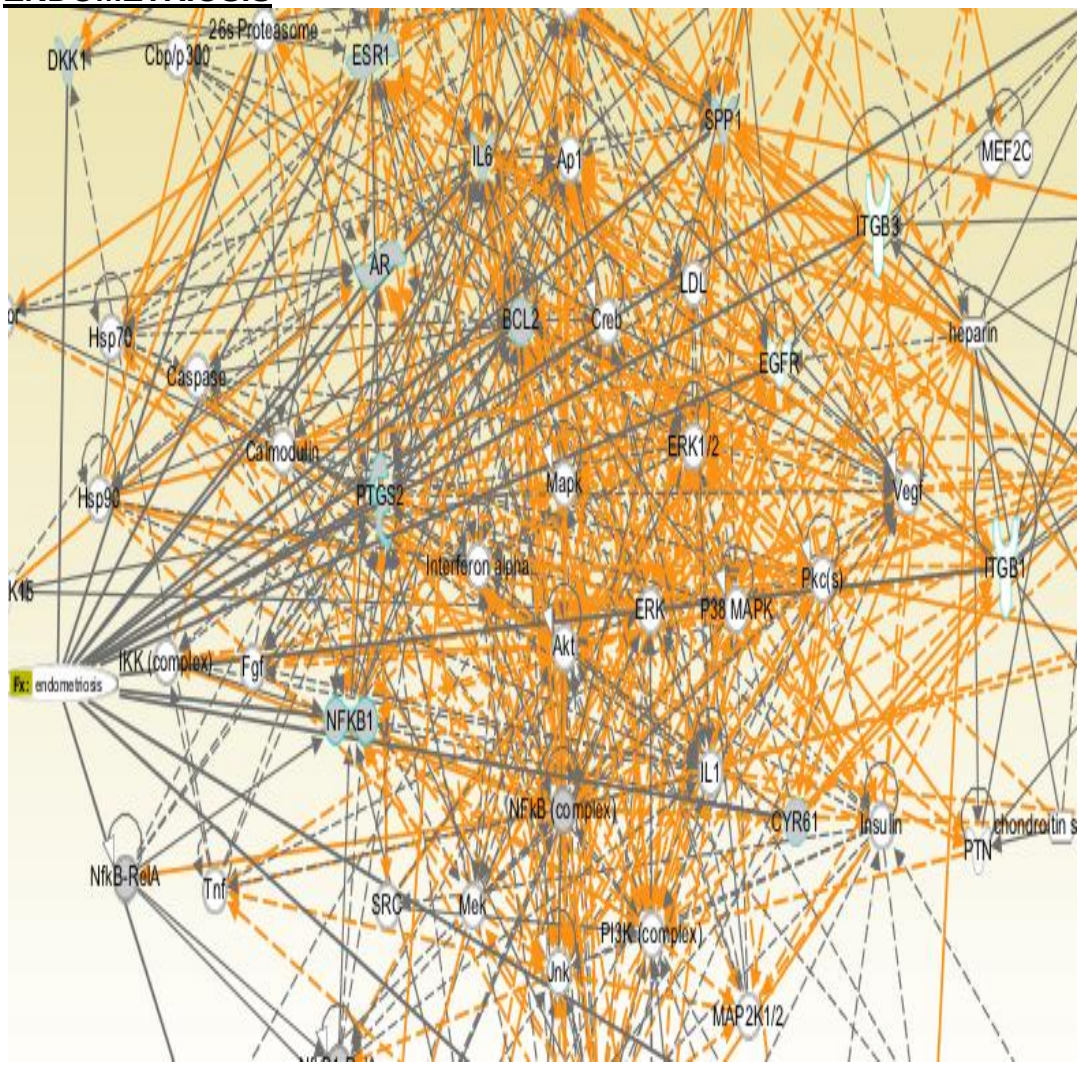


IMAGE 3.48 shows FOXD3 (red circle) involved in the interlinked mapping system in Ingenuity. The inserted figures from our list in the mapping system are colored in grey.

Even though it is not placed centrally, the map shows how E2F4 (another transcription factor) binds to FOXD3. The transcription E2F4 linked to FOXD3, increases the activation of BCL2 and has it's binding increased by IL-6. As well, FOXD3 binds to another transcription factor FOXA1, which is part of the same family of Fork-head factors. Ingenuity displays how FOXA1 binds to Androgen Receptor, as well as increasing the expression of ESR1. Therefore it is clear, from the above image and our Ingenuity network, FOXD3 is a key major transcription factor in regards to groups of up regulated players in endometriosis.

A more detailed explanation will be provided in Chapter 5:Discussion into how FOXD3 and some of the key players are related to endometriosis. We conducted immunohistochemistry lab-work validate our findings and observe any involvement of FOXD3 in the endometrium of patients with and without endometriosis (please refer to Chapter 2: Immunohistochemistry Methodology).

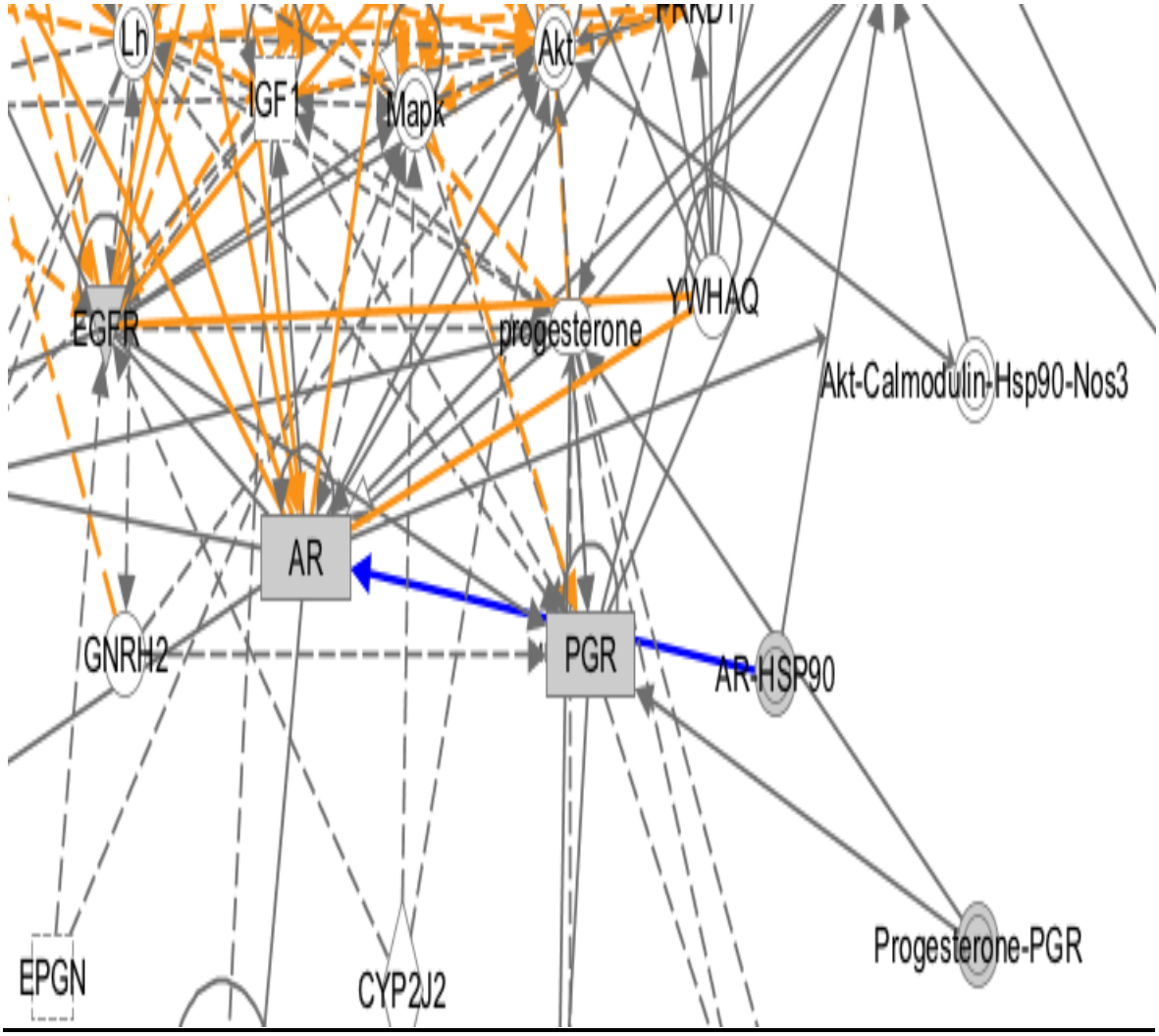
IMAGE 3.49
IMAGE SHOWING THE RELATIONSHIP OF ANDROGEN RECEPTOR AND OTHER UP REGULATED GENES/MOLECULES/PROTEINS ETC IN ENDOMETRIOSIS



Androgen receptor (AR) is up regulated in endometriosis (Fig 3.47). Our network (Image 3.49) showed that it is also a central figure in relation to other up regulated functions. Danazol, which is an agonist of Androgen Receptor, is an approved treatment in patients with endometriosis (explained in Chapter 1: Introduction). Also, the IPA mapping system shows that other functions that are controlled by FOXD3, such as NFKB1, are also seen to bind to AR (Androgen Receptor) in a cell-free system. Also as outlined in figure 3.50, there is an involvement of Progesterone Receptor (PR) within the network of genes up regulated in endometriosis.

Therefore based on these findings, we also conducted immunohistochemistry labwork to confirm the activity of AR and PR in endometriosis, of which the involvement previous authors have already studied. (See Chapter 5: Discussion)

IMAGE 3.50
IMAGE SHOWING RELATIONSHIP OF PROGESTERONE RECEPTOR WITHIN THE IPA INTERFACE NETWORK INVOLVING UPREGULATED GENES IN ENDOMETRIOSIS



IMMUNOHISTOCHEMISTRY DATA

FOXD3 Immunohistochemistry data.

The slides were blinded prior to intensity scoring of the basal layer glands, functional layer glands, in the endometrial stroma and luminal epithelium. Scoring was assessed as absent (0), weak (1), moderate (2), strong (3) or very strong (4). The slides that had basal layer glands were full thickness samples (explained in Chapter 3- Immunohistochemistry Methodology). After independently scoring the slides, the scores and slides were double checked by my experimental supervisors.

FIGURE 3.51- Micrograph to show an example of the intensity staining of fertile control (Rabbit IgG) weak staining in the basal layer gland of endometrium during the window of implantation

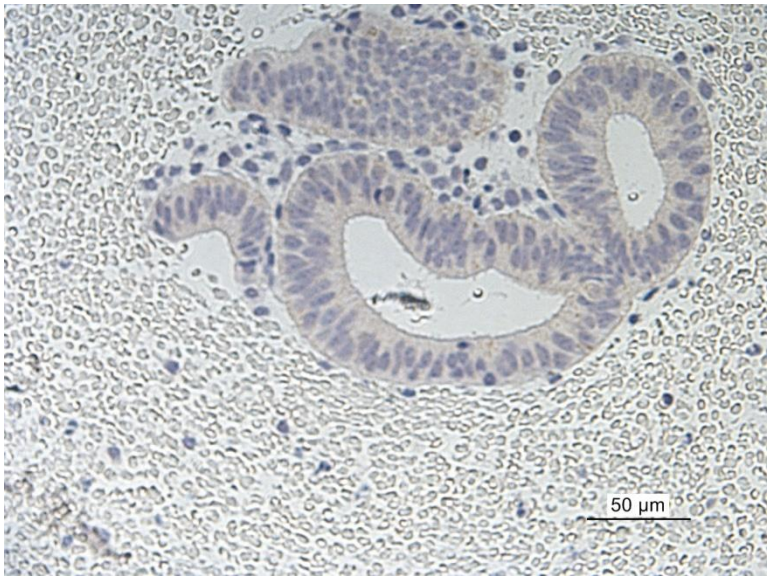


Figure 3.52- Micrograph to show an example of FOXD3 weak staining in the basal layer gland of endometrium.

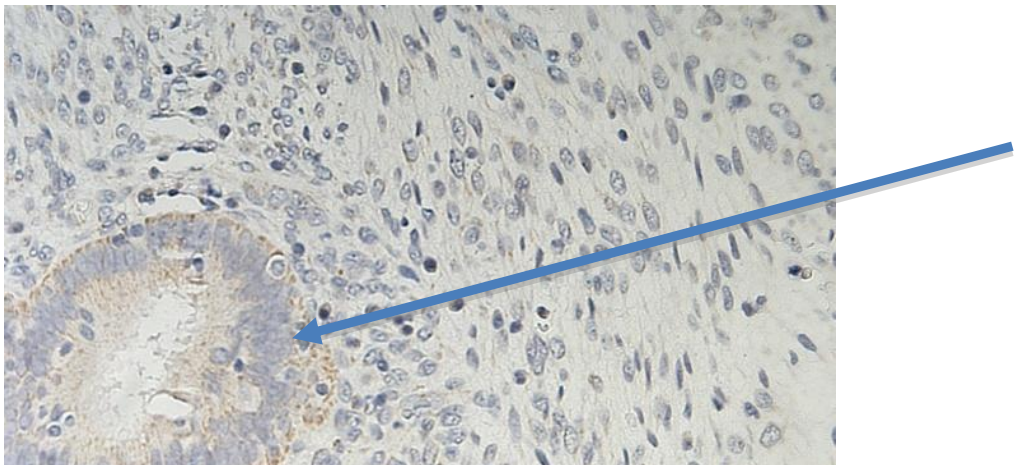


Figure 3.53- Micrograph to show an example of FOXD3 strong staining in the functional layer gland of endometrium

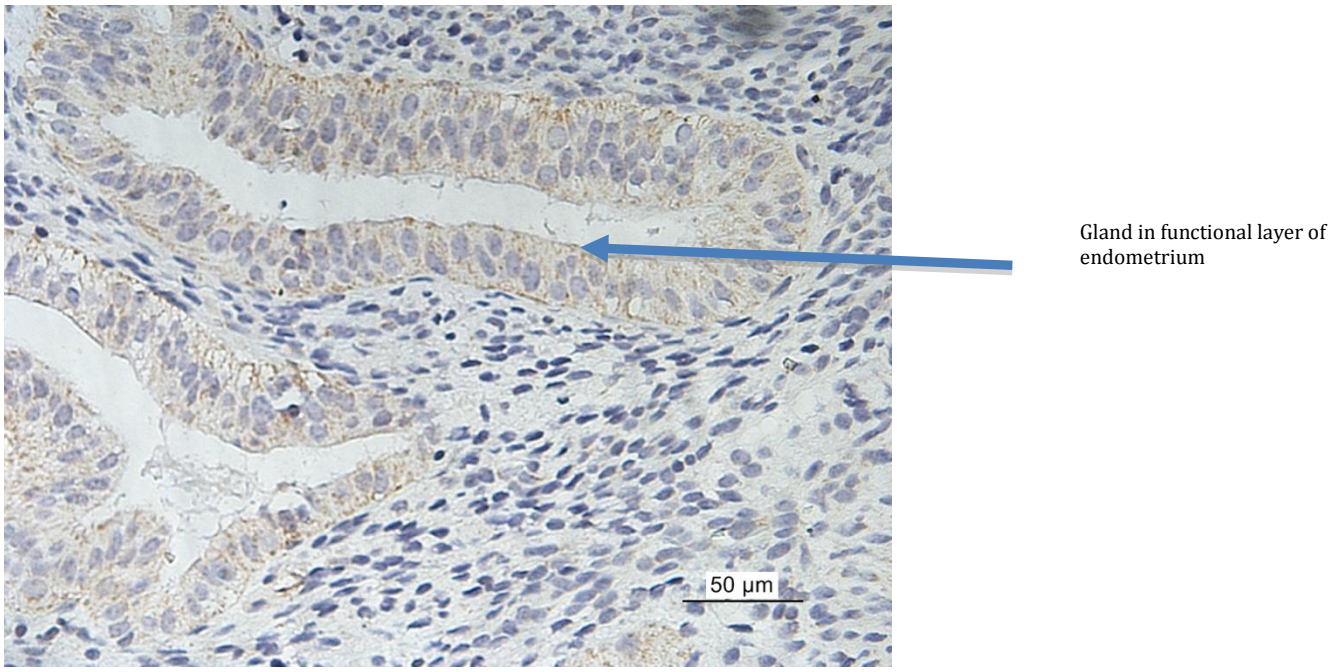
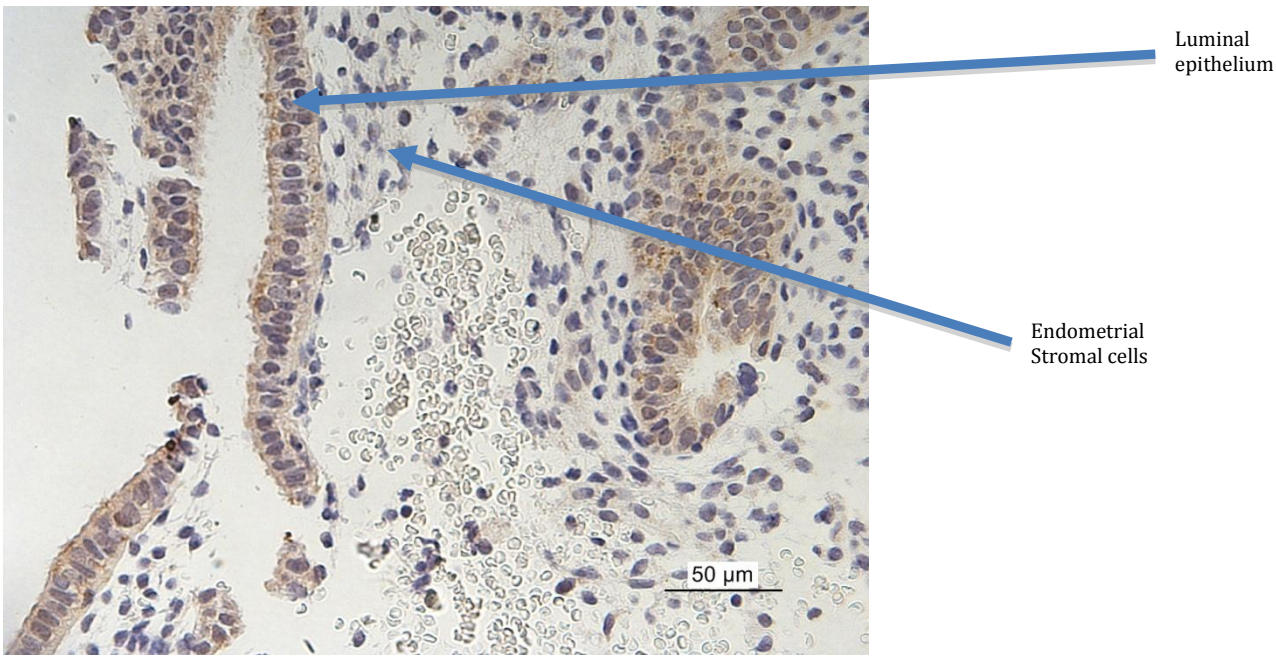


Figure 3.54- Micrograph to show an example of FOXD3 very strong staining in the functional layer gland and luminal epithelium of the endometrium



Reference images were captured after reviewing all of the slides and each slide was compared to the representative photos for each score. These scores were entered into GraphPad Prism version 5 in order to create Scatter-graphs and SPSS Statistical Software to discover whether there was any statistical significance in our findings.

FIGURE 3.55
A scatter graph showing the comparison of the intensity staining of FOXD3 in the glands of the functional layer in the endometrium of women with endometriosis to normal fertile patients, at two different stages of the menstrual cycle.

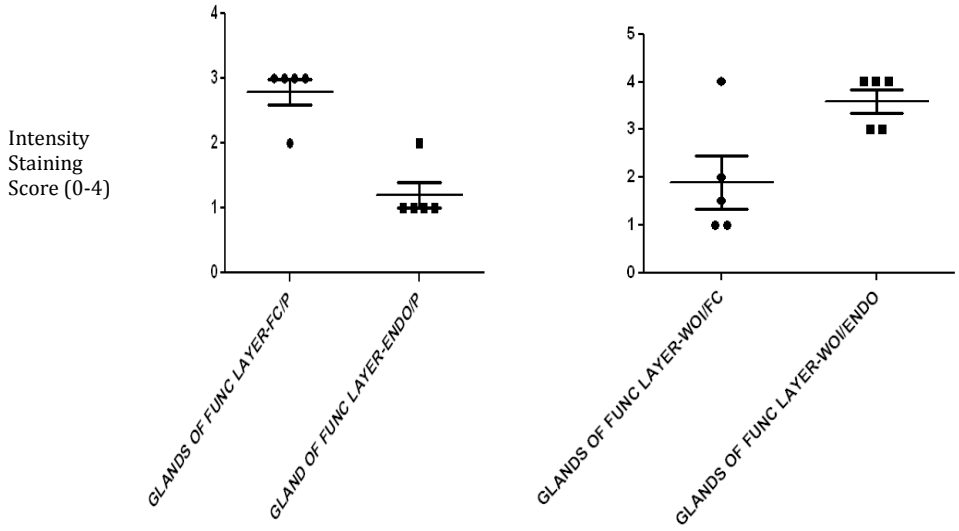


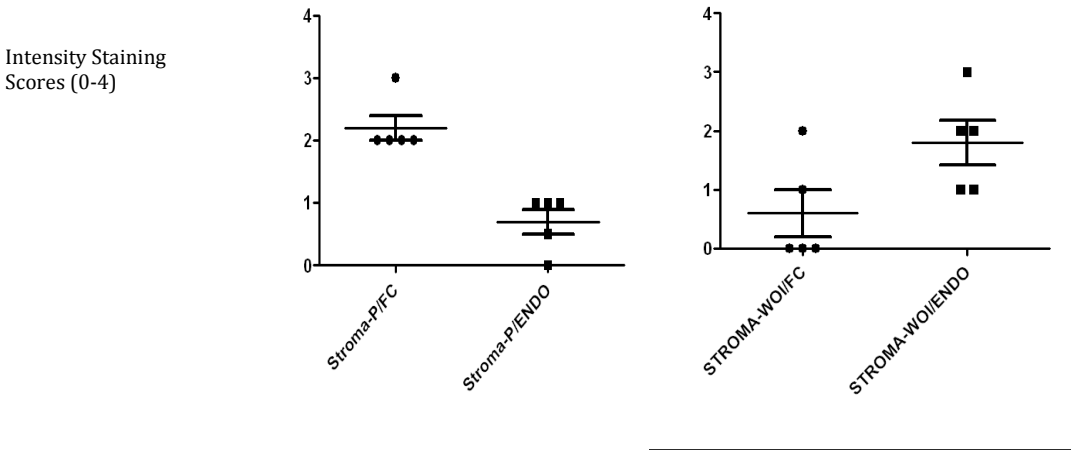
Figure legend for all scatter graphs below.
STAGE
WOI- Window of Implantation of menstrual cycle
P- Proliferative phase

SLIDE
ENDO- Endometriosis sample
FC- Fertile Control sample

The two graphs show increased staining intensity of FOXD3 in the functional glands of fertile control patients compared to the functional glands of the endometriosis patients. However, in the window of implantation, there is a higher staining of FOXD3 in the functional glands of endometriosis patients compared to that of the fertile control patients.

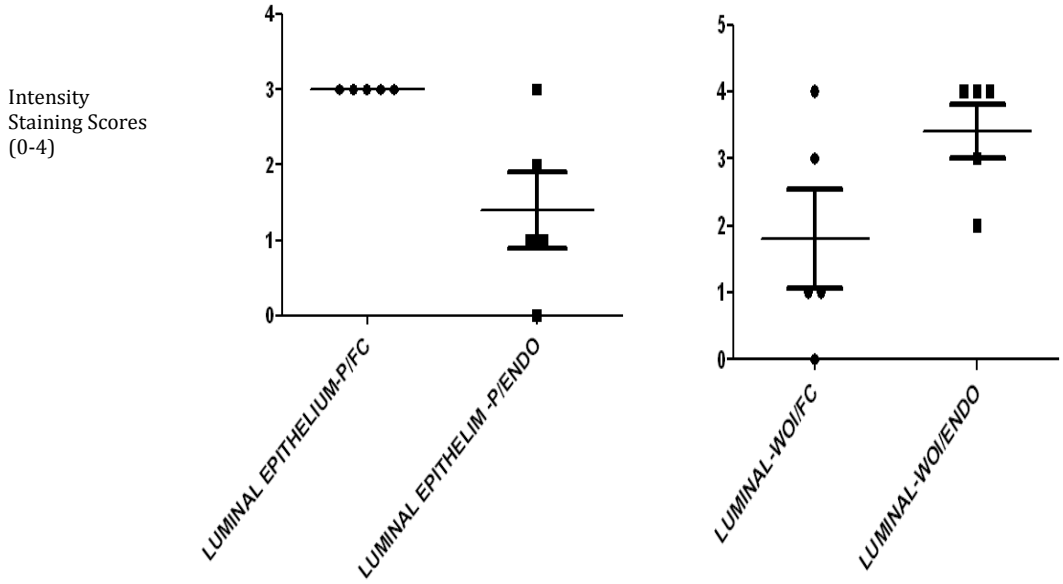
FIGURE 3.56

A scatter graph showing the comparison of the intensity staining of FOXD3 in the endometrial stroma of women with endometriosis to normal fertile patients, at two different stages of the menstrual cycle.



The above graphs show increased staining of FOXD3 in the endometrial stroma in the proliferative phase in fertile patients compared to patients with endometriosis. However in the window of implantation, the staining of FOXD3 is increased in the endometrial stroma in patients with endometriosis compared to fertile control patients. As can be seen, the staining in the stroma of FOXD3 was not as strong as that in the glands. This told us that FOXD3 did not occupy the stromal area as strongly as it did in the epithelium and glandular area, so interaction with antigenic sites in the sample was weaker, especially in patients with endometriosis.

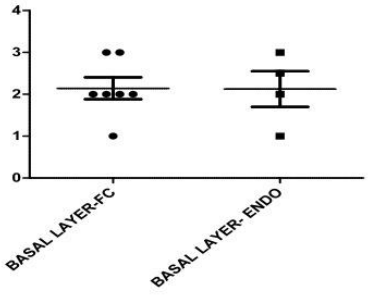
FIGURE 3.57
A scatter graph showing the comparison of the intensity staining of FOXD3 in the luminal epithelium of the endometrium of women with endometriosis to normal fertile patients, at two different stages of the menstrual cycle.



The above graphs show increased staining of FOXD3 in the luminal epithelium of the endometrium in the proliferative phase in fertile patients compared to patients with endometriosis. However in the window of implantation phase, the staining of FOXD3 is higher in the luminal epithelium of the endometrium of patients with endometriosis compared to fertile control patients.

FIGURE 3.58
A scatter graph showing the comparison of the intensity staining of FOXD3 in the basal glands of the endometrium of women with endometriosis to normal fertile patients.

A graph to show the intensity staining of FOXD3 in the basal layer of the endometrium in women with endometriosis compared to normal fertile patients.



The above graph shows there is little difference in the staining of FOXD3 in the basal glands in the endometrium of patients with endometriosis compared to normal fertile control patients. However, it must not be forgotten that there was 7 full thickness fertile control patient samples compares to 4 full thickness endometriosis samples.

STATISTICAL ANALYSIS OF FOXD3 STAINING FINDINGS

In using a non-parametric Mann-Whitney U test, it was found there was significant statistical difference in the glandular (basal + functional gland) FOXD3 staining in endometriosis patients in the proliferative phase compared to the window of implantation ($p=0.008$). It was also found that there was significant statistical difference in FOXD3 staining in fertile control patients in the endometrial stroma in the proliferative phase compared to the window of implantation ($p=0.03$).

It was also found when comparing fertile control to endometriosis in the proliferative phase, FOXD3 staining was statistically significant in the luminal epithelium ($p=0.03$), the endometrial stroma ($p=0.008$) and glandular areas ($p=0.008$).

However, FOXD3 staining is not significantly significant in the Window of Implantation in any of the 4-endometrial compartments when comparing fertile patients to endometriosis patients.

FIGURE 3.59
A micrograph to show the staining of FOXD3 in the stromal cells and glands in the endometrium of a normal fertile control patient (left) compared to a endometriosis patient (right), during the window of implantation.

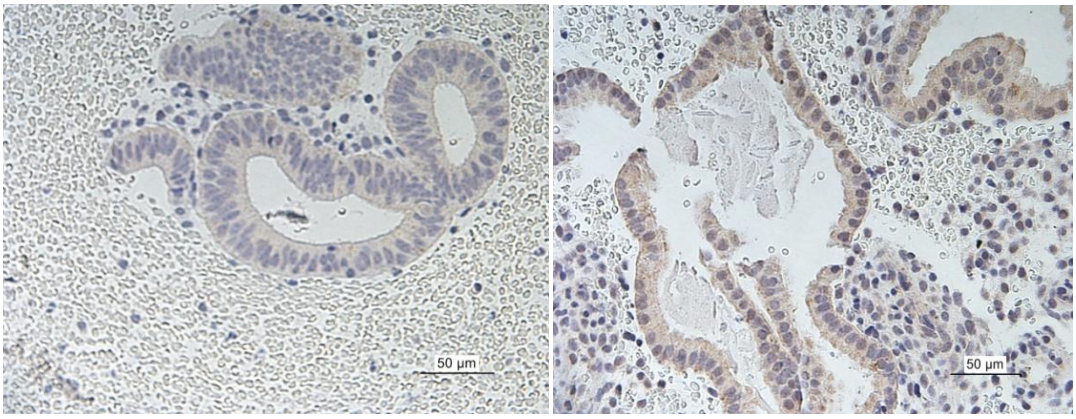
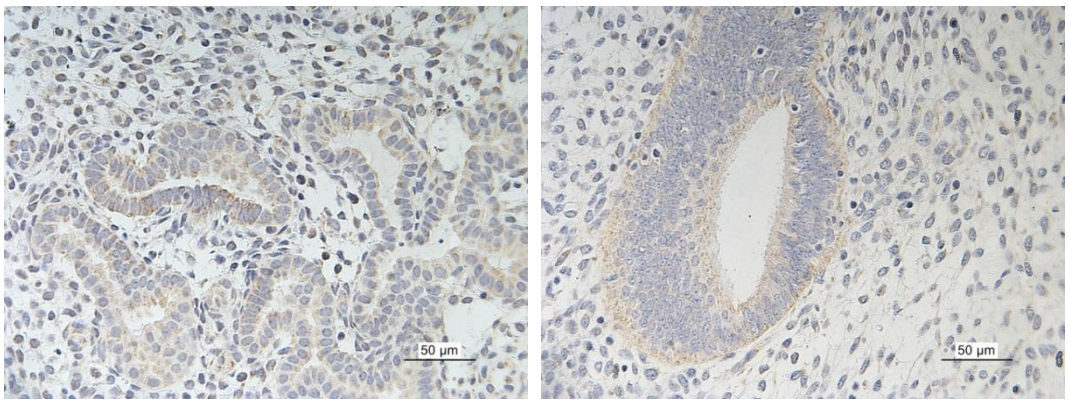


FIGURE 3.60
A micrograph to show the comparison of FOXD3 staining in the glandular area of endometrium in a normal fertile control patient (left) to an endometriosis patient (right), in the proliferative phase.



As can be seen in Figure 3.58, there is a clearer increased intensity of staining in the glandular and stromal area in the endometriosis patient compared to the normal fertile control patient.

The darker intensity of staining indicates that in the endometrial sample, there were more specific antigens in the endometrial tissue for the FOXD3 antibody to bind to.

Therefore, as can be seen, there is a significant difference in the presence of FOXD3 in patients with endometriosis compared to normal fertile patients.

The increased expression of FOXD3 is explained in Chapter 4: Discussion.

PROGESTERONE RECEPTOR (PR) IMMUNOHISTOCHEMISTRY RESULTS

The slides were blinded prior to intensity scoring of the basal layer glands, functional layer glands, endometrial stroma and luminal epithelium. Scoring was assessed as absent (0), weak (1), moderate (2) or strong (3). The slides that had basal layer glands were full thickness samples (explained in Chapter 3- Immunohistochemistry Methodology).

FIGURE 3.61
A scatter graph showing the comparison of the intensity staining of Progesterone receptor in the glands of the functional layer in the endometrium of women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

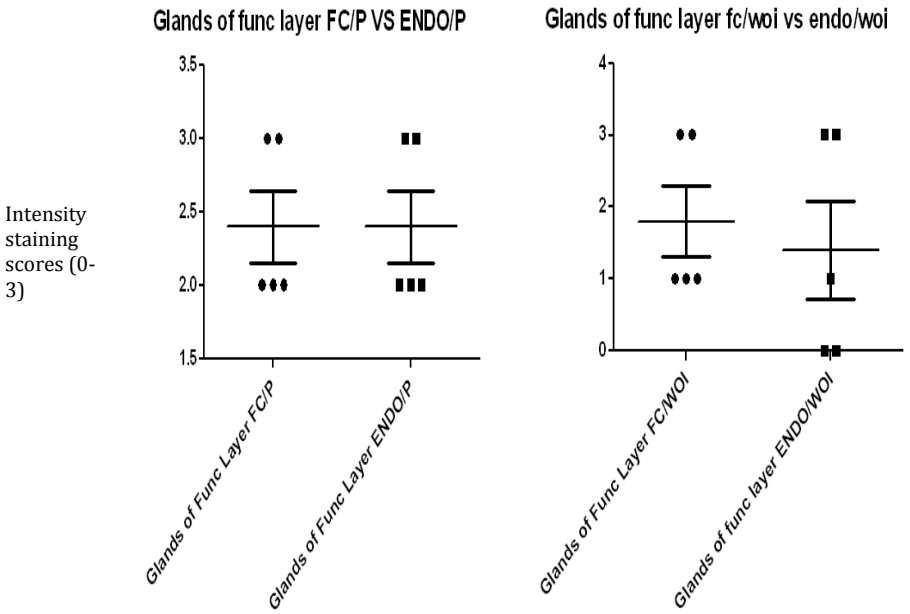


Figure legend for all scattergraphs below
STAGE
WOI- Window of Implantation of menstrual cycle
P- Proliferative phase

SLIDE
ENDO- Endometriosis sample
FC- Fertile Control sample

FIGURE 3.62
A scatter graph showing the comparison of the intensity staining of Progesterone receptor in the endometrial stroma of women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

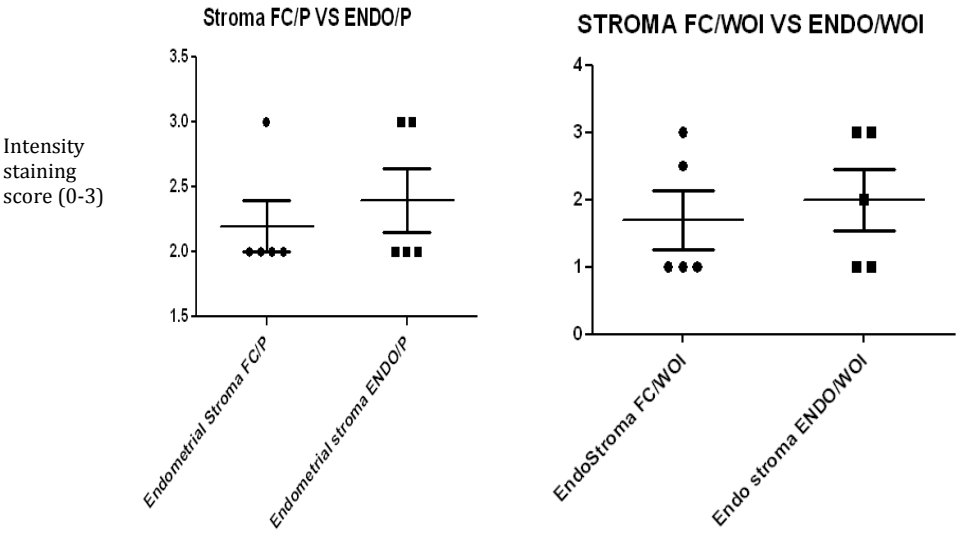


FIGURE 3.63
A scatter graph showing the comparison of the intensity staining of Progesterone receptor in the luminal epithelium of the endometrium of women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

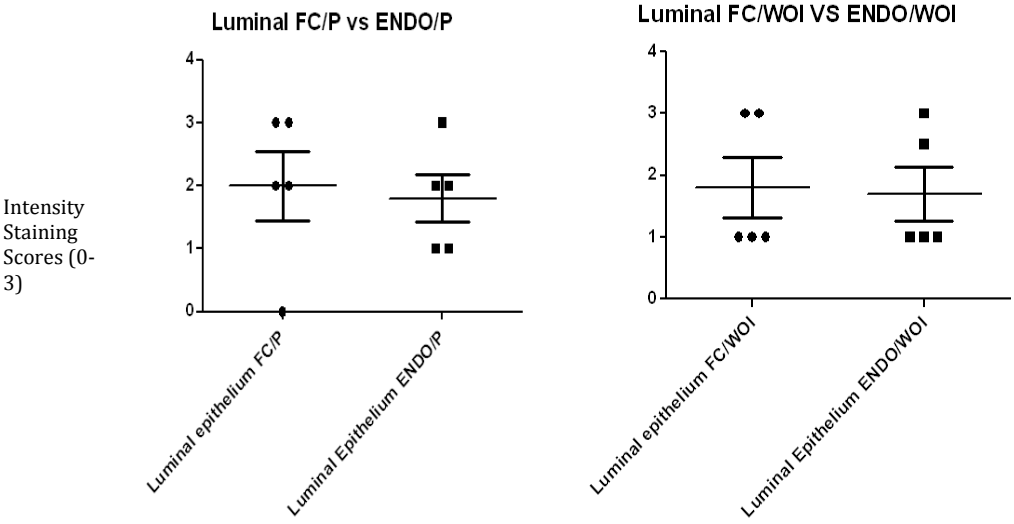
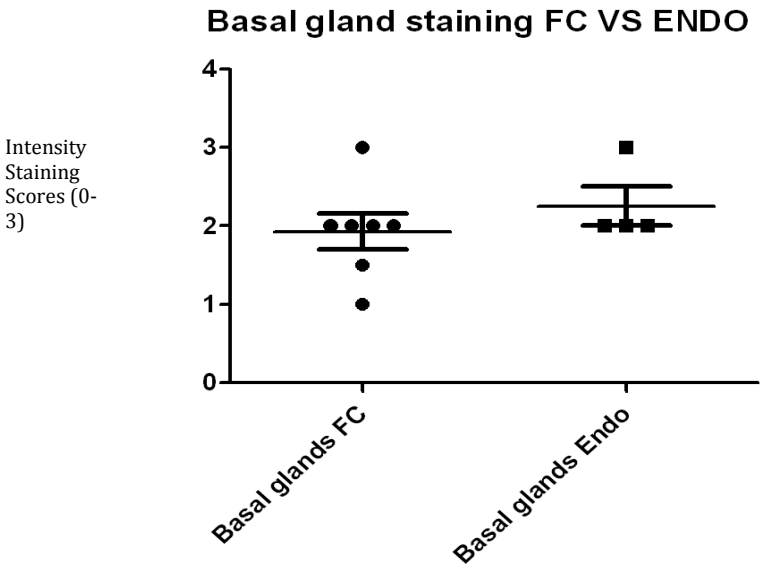


FIGURE 3.64
A standard deviation scatter graph showing the comparison of the intensity staining of Progesterone receptor in the basal glands of the endometrium of women with endometriosis (ENDO) to normal fertile control (FC) patients.



Statistical Significance of Progesterone Receptor staining intensity in patients in endometriosis compared to normal fertile control patients

There was no statistical significance according to the SPSS stats in the progesterone receptor intensity staining in the endometrium at collectively or at any time point (proliferative or window of implantation) of endometriosis patients compared to normal fertile control patients. As well, there was no statistical significant difference between intensity staining of progesterone receptor in any of the 4 compartments within the endometrium in the two groups. Therefore, it is clear from the statistical significance data and the graphs listed, we could not conclude there to be a difference in the expression of Progesterone Receptors in patients with endometriosis compared to normal fertile control patients.

ANDROGEN RECEPTOR (AR) IMMUNOHISTOCHEMISTRY RESULTS

The slides were blinded prior to intensity scoring of the basal layer glands, functional layer glands, and endometrial stroma and luminal epithelium. Scoring was assessed as absent (0), weak (1), moderate (2) or strong (3). The slides that had basal layer glands were full thickness samples (explained in Chapter 2- Immunohistochemistry Methodology).

FIGURE 3.65
A scatter graph showing the comparison of the intensity staining of Androgen receptor in the glands of the functional layer in the endometrium of women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

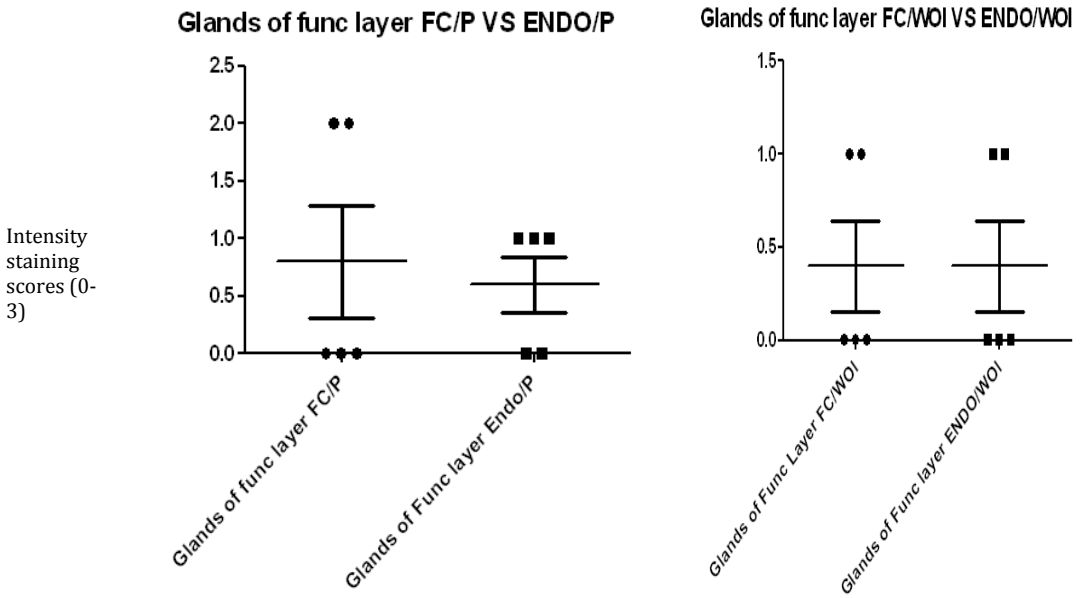


Figure legend for all scattergraphs below

STAGE

WOI- Window of Implantation of menstrual cycle
P- Proliferative phase

SLIDE

ENDO- Endometriosis sample
FC- Fertile Control sample

FIGURE 3.66
A scatter graph showing the comparison of the intensity staining of Androgen receptor in the luminal epithelium of the endometrium of

women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

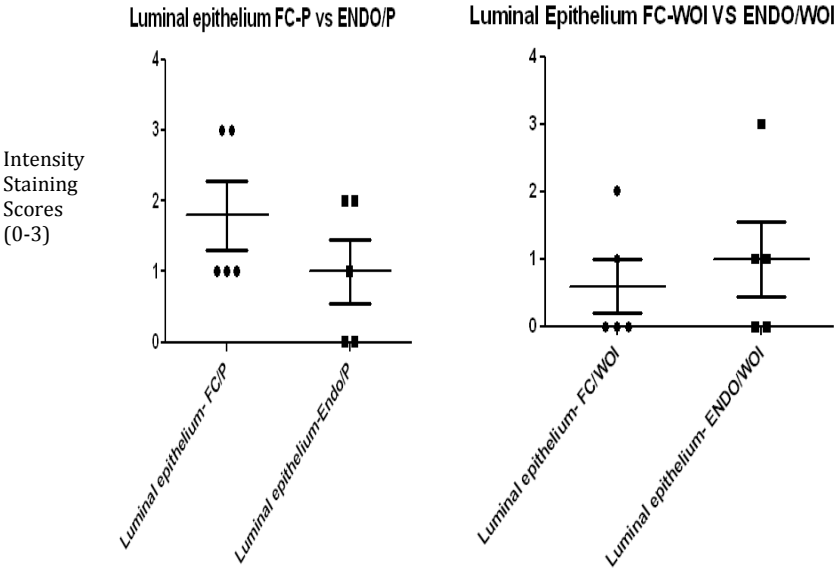


FIGURE 3.67
A scatter graph showing the comparison of the intensity staining of Androgen receptor in the endometrial stroma of women with endometriosis (ENDO) to normal fertile control (FC) patients, at two different stages of the menstrual cycle.

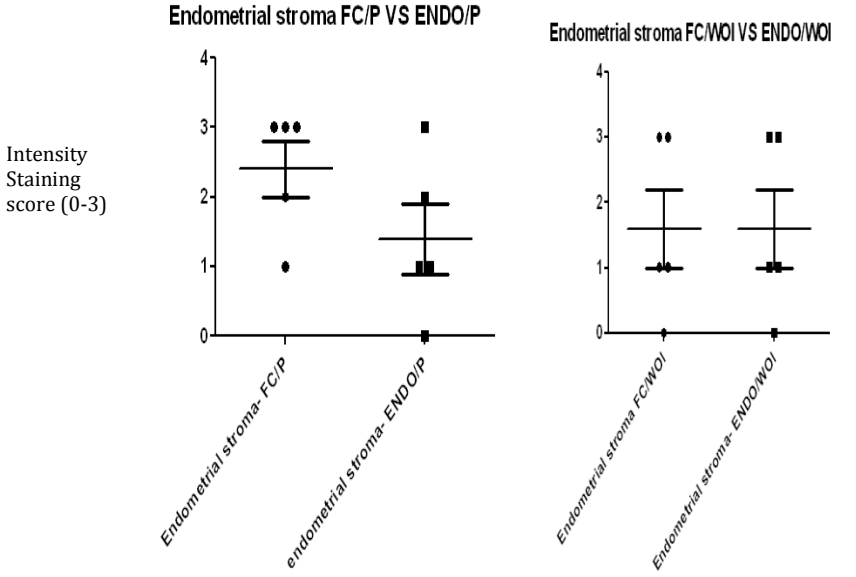
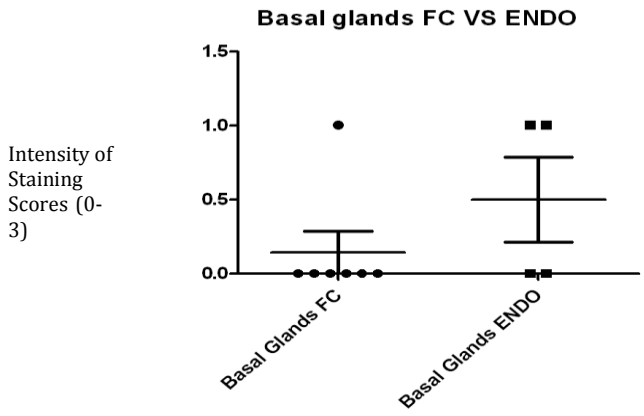


FIGURE 3.68
A scatter graph showing the comparison of the intensity staining of Androgen receptor in the basal glands of the endometrium of women with endometriosis (ENDO) to normal fertile control (FC) patients.



<u>Statistical Difference of Androgen Receptors in the endometrium in patients with endometriosis compared to normal fertile control patients</u>

Similarly to the significance of Progesterone Receptor staining, there was no statistical significance according to the SPSS stats in the androgen receptor intensity staining in the endometrium at collectively or at any time point (proliferative or window of implantation) of endometriosis patients compared to normal fertile control patients.

As well, there was no statistical significant difference between intensity staining of androgen receptor in any of the 4 compartments within the endometrium in the two groups. Therefore, it is clear from the statistical significance data and the graphs listed, we could not conclude there to be a difference in the staining of androgen Receptors in patients with endometriosis compared to normal fertile control patients.

TABLE 3.69
A TABLE OF DEMOGRAPHICS OF PARTICIPANTS (ENDOMETRIOSIS AND FERTILE CONTROL PATIENTS) USED IN THE STUDY

Sample	Age	Parity	BMI (Body Mass Index)	Cycle length	Endometriosis stage	
SPCE29	49	2	32.7	28		
SPCN11						
SPCN37	40	1	30.1	28		
SPCN40-2	37	2	26.5	28		
RM260	36					
SPCE21-1	33	2	20.3	28	1 2	
SPCE2	45	0	32	28		2
SPCE17	33	0	22.2	32		3
SPCE23-1	41	2	27.8			
SPCN23	42	4	35.8	28		
EN55	36	1	31.4	28		3
EN39	41	1		28		4
SPCN27	30	3	28.1	30		
EN56	30					
SPCN17	32	4	22.8	28		
SPCN32-1	28	1	29.7	28		
EN09	35	0	24	26		
SPCN31	40	2	34.3	28		
SPCN20	43	0	26.7	28		
SPCN33	44	2	24.3	28		
SPCE30	38	0	23.3	28		1

From our results, we found there was significant difference in the expression of FOXD3 in patients with endometriosis compared to those without. We can predict genes controlled by this transcription factor up regulated from our bioinformatics study, were increased in these patients.

The immunohistochemistry procedures were able to provide a definitive confirmation of FOXD3 being present in patients with endometriosis. Using the table of demographics listed above of patients as well as earlier bioinformatic findings, we explained in the next chapter (Chapter 4: Discussion) why FOXD3 staining was significantly different in patients with endometriosis compared to fertile control patients. With no relevant studies on FOXD3 and its involvement in the pathology of endometriosis, we hoped to

provide a possible breakthrough in future work for diagnostic and therapeutic purposes.

CHAPTER 4: DISCUSSION

In Chapter 3, we showed how bioinformatics and computational technology were used to discover key genes up regulated in various pathological processes during endometriosis. We were able to discover some common up regulated genes/proteins in these different cellular aberrations and relate this expressional pattern to other conditions. The interlinking of the identified genes in the pathological processes related to endometriosis will be explained in this chapter.

With the use of bioinformatics, we identified FOXD3 in the pathogenesis of endometriosis. It was found that it played a regulatory role in a large number of genes that have been already published and known to be associated with endometriosis. In validating our bioinformatics findings, we conducted immunohistochemistry (IHC) work to discover whether FOXD3 was expressed in the human endometrium, and aberrantly expressed in the eutopic endometrium of patients with endometriosis.

Our bioinformatics findings also showed Androgen receptors (AR) and Progesterone Receptors (PR) to be involved in the interlinking pathways of genes during endometriosis. Previous authors had suggested AR and PR to be involved in the pathogenesis of endometriosis with the use of IHC laboratory work. Therefore we conducted an IHC study of the same endometrial samples to investigate if there is a difference in the expression of AR and PR between the normal endometrium and the eutopic endometrium of patients with endometriosis.

Our extended IHC showed that FOXD3 is expressed in the human endometrium for the first time. There was a statistically significant difference in the semi-quantitative staining scores between patients with endometriosis and normal fertile control patients in the glandular areas, luminal epithelium and stromal area in the endometrium during the proliferative phase of the menstrual cycle. On the contrary, we could not find a significant difference in

the staining of androgen and progesterone receptors in the endometrium of patients with endometriosis compared to normal fertile control participants.

4.1 Discussion of the main Bioinformatics findings

As seen in Chapter 3: Data and Analysis, the use of bioinformatics made it possible to identify that there are certain genes that appear to be commonly up regulated in endometriosis and its related pathological processes. With my bioinformatics, we found IL-8, StAR, CXCR4, PTGS2, SPP1 and CXCR4 were seen be to be very strongly up regulated in the various pathological events related to endometriosis. The genes displayed a link between common processes. From the meta-analysis provided by Genevestigator, we found similar expressional patterns of SPP1, IL-8, StAR and CXCR4 in gastric cancer. I will therefore discuss each of the individual genes/proteins as well as the available evidence for their involvement in endometriosis and other pathological conditions and how they may be involved in the disease process.

4.1.1-The expression of Secreted Phosphoprotein-1 (SPP1)

SPP1 was seen to be a gene strongly up regulated in endometriosis and also within the related pathological process calcium signalling. SPP1, also known as osteopontin (OPN) was regulated by FOXD3 in its transcriptional activities. (117)

SPP1 has been seen to be important in bone remodelling and anchoring osteoclasts to the mineral part of the matrix in the bone. (117) It is also important in the function of the immune system, inhibiting the production of the cytokine IL-10 (Th2 cytokine), therefore promoting the function of the Th1 cytokine and influencing a cell-mediated immunity.

There is available evidence that has studied the expression of SPP1 (OPN) within endometriosis. Cho et al in 2009 showed how osteopontin (SPP1) expression was increased in patients with endometriosis and how plasma levels of SPP1 may be used as a non-invasive marker for the diagnosis of

endometriosis. (117) Odagiri et al in 2007 also displayed how SPP1 played an important role in the pathogenesis of endometriosis. (118) Odagiri found that OPN was stained strongly in the glandular areas of the endometrium in both human and rat endometriotic tissue samples. (118) However in contrast, Wei et al in 2009 showed that there was a decreased expression of SPP1 in the late secretory phase in patients with endometriosis, indicating that this may be linked to the impaired endometrial receptivity in endometriosis patients. (119)

SPP1 is a common protein that regulates macrophages during an inflammatory response at sites of chronic inflammation. We suggest that OPN is largely up regulated in the endometrium due to the increase in concentration of macrophages and other cytokines in the pelvic cavity. (117) The increased macrophage concentration is important in processing the foreign invading endometrial cells, and producing antigen-presenting cells for the antibodies. Therefore OPN may be strongly up regulated in endometriosis due to the increased macrophage production in the pelvic peritoneum as well as the altered immune response that occurs.

There is available evidence to suggest a link between OPN and calcium signalling. You et al in 2001 investigated the regulation of OPN by oscillatory fluid flow via intracellular calcium mobilization and the activation of mitogen-activated protein kinase in osteoblasts. (120) The study outlined how OPN regulation would increase after a 2-hour oscillatory fluid flow involving calcium ions. (120) We understand that an increase in intracellular calcium (Ca2+) and mitogen activated protein kinase (MAPK) during calcium signalling in the endometrium is therefore likely increase the regulation of OPN. Additionally Khoshniat et al in 2011 investigated how the Phosphate-dependent stimulation of OPN expression in osteoblasts through ERK1/2 pathway is modulated by calcium. (121) This study showed how calcium and it's signalling pathway played a role in the functioning of osetoblasts, increasing the expression of Phosphate and OPN. (121)

We can therefore predict in the uterus, the flow of calcium ions and its signalling pathway inducting the contraction of myocyte cells in the myometrium may up regulate SPP1 during endometriosis.

We also found SPP1 (OPN) to be very strongly up regulated in gastric cancer. Studies such as that by Junilla et al in 2011 have shown how there has been over expression of SPP1 in metastasized tumours. (125) SPP1 is an important mediator in stress response, cell adhesion and angiogenesis and therefore is seen to play a pivotal role in the metastasis and angiogensis in cancer. (122) Chang et al in 2011 studied how an increased gastric expression of SPP1 (OPN) by Helicobacter Pylori infection may correlate with a more severe gastric inflammation and intestinal metaplasia. (122) In gastric cancer, inflammation of the intestinal epithelium progresses to metaplasia eventually resulting in adenocarcinoma of the cells.

We can predict SPP1 to carry out similar roles in gastric cancer to endometriosis through its inflammatory, adhesive and angiogenesis functions.

4.1.2- The expression of Interleukin-8 (IL-8)

According to our bioinformatics findings, IL-8 was strongly up regulated in proliferation of the endometrium, retrograde menstruation and inflammation in with endometriosis.

IL-8 is a cytokine immune factor that induces neutrophil chemotaxis at site of inflammation. Past studies have confirmed our findings of IL-8 playing an important role in the proliferation of the endometrium in endometriosis. (123) Arici et al in 1998 showed that IL-8 directly stimulates the growth and proliferation of endometrial cells. (123) The study showed that there was an increase in the proliferative activity of endometrial cells in correspondence to the in vivo concentration of IL-8. Therefore the increase in the concentration of IL-8 in the peritoneal fluid of endometriosis patients provides a plausible explanation of there being an involvement in the growth of menstrual debris from retrograde menstruation. Another study from Ulukus et al in 2009 investigated IL-8 levels to be highest in the eutopic endometrium of patients with endometriosis during the proliferative phase. (124) Refluxed endometrial cells that attach to the extracellular matrix and outside the pelvic peritoneum are likely to secrete higher levels of IL-8. (123) In response, granulocytes are recruited and an inflammatory reaction will occur.

The bioinformatics data from Genevestigator also showed IL-8 to be up strongly up regulated in both gastric cancer and endometriosis. A study by Bartchewsky et al in 2009 found that there were higher levels of IL-8 and COX-2 detected in the gastric mucosa of patients. (126) Up-regulation of this interleukin occurred in correspondence to H-pylori causing cellular proliferation and gastric mucosal damage, resulting in the development of gastric adenocarcinoma. (126) Ju et al in 2010 showed that IL-8 associated with adhesion, migration, and invasion in gastric cancer cells. Similarly to our bioinformatics findings, Ju et al found IL-8 to be over expressed in gastric tumour cells, and discovered it to promote cell adhesion in endothelial cells as well invasion in gastric cancer. (127)

Being an important interleukin during the inflammatory processes, we can suggest IL-8's function of being involved in the proliferative and reflux activity of endometrial cells can be compared to the function of the mediator during it's interaction with H-Pylori in the gastric mucosa. Therefore a similarity can be predicted in the strong up regulation of IL-8 during the pathogenesis of endometriosis and in gastric adenocarcinoma.

4.1.3- The expression of CXCR4

CXCR4 was up regulated in the altered immune responses and oestrogen responsiveness during endometriosis according to our bioinformatics findings.

The CXCR4 gene encodes a specific CXC chemokine receptor and endometriosis is a process in which local inflammatory factors are activated. Studies such as that by Ruiz et al in 2010 have reported CXCR4 levels to be higher in endometriosis patients and this could be responsible for the survival of ectopic endometrial cells implanted. (128)
Many studies have identified a link between CXCR4 signalling and Oestrogen receptor signaling pathways in breast cancer. Furuya et al in 2007 investigated how CXCR4 signaling contributed to the Oestrogen-receptor dependent gene expression and therefore the growth of breast cancer cells. Furuya also predicted that oestrogen receptor expression was activated by

SDF-1 in the presence of CXCR4. (129) Other studies have also confirmed 17β-estradiol (E2) to increase the expression of CXCR4 and CXC12 in oestrogen receptors. (129)

With endometriosis being an oestrogen dependent disease and requiring the hormone for growth of ectopic lesion, we can predict CXCR4 to be up regulated in the same way as it is in breast cancer.

Breast cancer, like endometriosis, is an oestrogen dependent disease and the growth of cancer cells is dependent on oestrogen receptor levels. However Ruiz et al found CXCR4 to be down-regulated by ovarian steroid hormones in endometrial epithelial cells. (128) Therefore, from our bioinformatics findings, we can predict CXCR4 to be up regulated in patients with endometriosis due to an altered immune response (involvement of inflammatory mediators).

4.1.4- The expression of Steroidogenic acute regulatory protein (StAR)

The StAR protein was observed as being up regulated during endometriosis as well being strongly up regulated in gastric cancer. Studies have shown the levels to be highest in the ectopic implants of early endometriosis. (130) StAR is a transport protein that regulates cholesterol transfer within the mitochondria and it is a rate-limiting step in oestrogen production and the production of other steroid hormones. (130) Tsai et al in 2001 showed how StAR expression in ectopic endometriotic tissue implants leads to increased peritoneal progesterone production, which is an important part of the pathology of endometriosis. (130) As well, Tian et al in 2009 studied how StAR expression is associated with the severity of endometriosis. (131) Immunohistochemistry was used in this study to evaluate the levels of StAR in the endometrium of women with and without endometriosis. (131) It was found the expression of StAR was higher in ectopic endometrial tissue and increasing expression correlated with the severity of the disease in the patients. (131) Therefore, with endometrial cells requiring oestrogen and progesterone in their growth and development, StAR is strongly up regulated, as it is a rate-limiting step in the production of these steroid hormones.

Akiyama et al in 1997 showed how CAB1, a gene expressed in gastric cancer cells, was seen to be homologous to StAR, therefore indicating its possible role in gastric cancer. (132) StAR is primarily present in steroid-producing cells such as the theca and luteal cells in the ovary. (132) Some metastatic carcinomas in the ovary such as the Krukenbergs tumor are derived from a primary malignancy in the gastrointestinal tract. (132)

With StAR playing an important role in cholesterol transport to the mitochondria, it could seen to be involved in the cellular survival and growth in both endometriosis implants and gastric cancer cells.

4.1.4- The expression of Prostaglandin-endoperoxide synthase 2 (PTGS2)

Our bioinformatics findings showed PTGS2 to be strongly up regulated within endometriosis, as well as during calcium signalling. PTGS2 which is also known as COX-2, is an enzyme encoded by the PTGS2 gene.

There is available evidence showing a link between the expression of PTGS2 and endometriosis. Liu et al in 2011 found PTGS2 promoter transcription activity to be increased in endometrial cells in endometriosis. (77) Cho et al in 2009 found that PTGS2 to be over expressed in the eutopic endometrium and ovarian endometriotic tissue in patients with endometriosis. (133) As explained in Chapter 1, PTGS2 (COX-2) is an important enzyme involved in the production of prostaglandin, one which contributes to pain and inflammation within endometriosis patients.

Our bioinformatics finding also showed that PTGS2 is up regulated during calcium signaling, a pathological process related to endometriosis. We propose that PTGS2 is up regulated in patients as prostaglandin production is increased during endometriosis. During endometriosis, pelvic pain and other related symptoms are likely to be a resultant of the increased contractions of the uterus. (76) Therefore, with a rise in prostaglandins in patients, this will bind to a G-Coupled receptor activating adenylyl cyclase and the protein kinase A (PKA). As a result, Ca 2+ ions influx and myometrial contractility

may occur. (76) Therefore up regulation of the enzyme PTGS2 may be related to calcium signaling in endometriosis.

4.2- Discussion of IHC findings

4.2- IHC OF FOXD3

Many studies have provided information on up or down regulated genes in endometriosis. It is difficult to understand the pathogenesis of endometriosis clearly due to the fragmented nature of the available evidence on the involvement of these genes in the disease. With plausible explanation being put forward in previous studies for the involvement of proteins such as IL-8, PTGS2, StAR, CXCR4 and SPP1 in endometriosis, breakthrough information into further possible theories of pathogenesis has failed to emerge.
Bioinformatics provided a useful tool to identify key transcription factors such as FOXD3 and FOXI1 that may regulate many of these separate pathological processes and genes.

From this data and the hypothesis generated from our bioinformatics study, we then progressed to test the involvement of one of the identified transcription factors in the human endometrium by carrying out a laboratory study. We focused on one of the common transcription factors, FOXD3 that seemed to regulate a large number of those genes that have already been reported to be involved in the pathogenesis of endometriosis and found a greater intensity of staining in the endometrium of patients with endometriosis compared to normal fertile control patients. The following is a list of genes/proteins up regulated in endometriosis that are controlled by FOXD3.

PTGS2	INSRR	DKK1	BCL2
STAR	ESR1	SPP1	RBP4
IL1A	NFKB1	IL6	CYR61

SMAD1	LAMA4	PTPN22	AR

4.2.1 FOXD3 expression within the normal endometrium

From our IHC findings, we found FOXD3 to be expressed in all areas of the normal endometrial during the proliferative phase. According to our work, there was a strong expression of FOXD3 in the luminal epithelium and glandular areas in the normal endometrium. The expression in endometrial stroma was not as strong as it was in the luminal epithelium and glandular locations, however there was moderate expression seen similarly in the basal layer glands.

We found there to be a decreased expression of FOXD3 from the proliferative to the secretory phase. There was seen to be a poor expression of FOXD3 in the endometrial stroma during the secretory phase, whilst the expression of FOXD3 in the luminal epithelium and glandular areas of the normal endometrium was much lower than it was in the secretory phase.

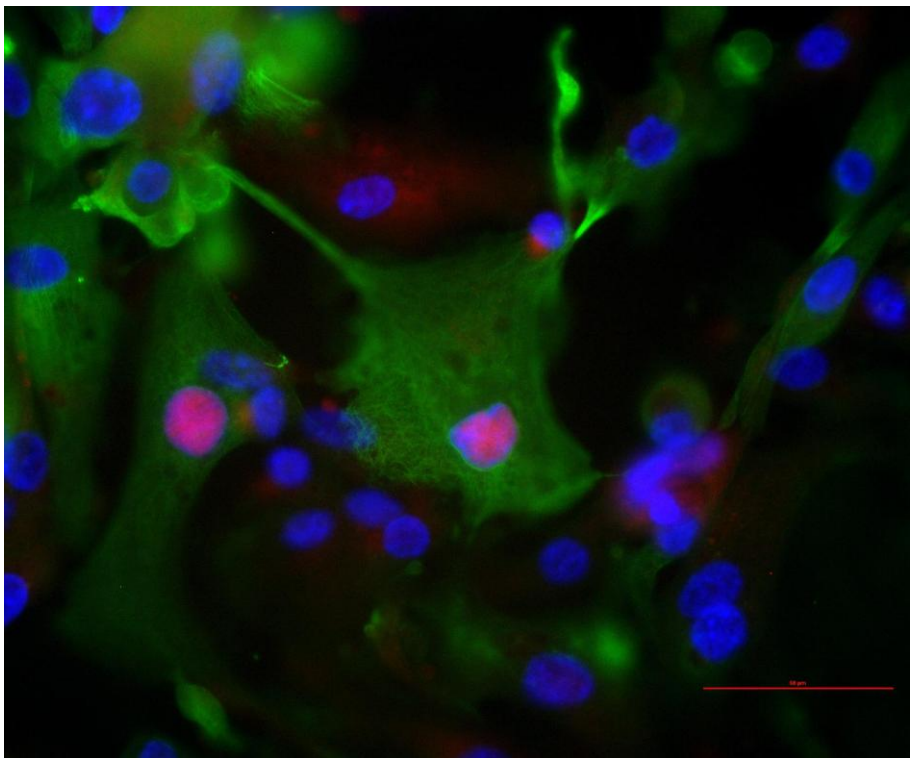
Expression of FOXD3 has been shown to promote neural crest induction and the transcription factor will be activated upon the emergence of neural crest cells, providing importance for the maintenance of stem cells. (134) Neural crest (NC) cells are progenitor multipotent cells that contribute to a number of derivatives. (135) Studies have shown FOXD3 to be detected in early embryogenesis within mice, playing an important role in maintaining the small population of cells in the epiblast (inner cell mass in mammals). (134)

FOXD3, a transcriptional regulator that is expressed in embryonic stem cells, is seen to play an important role in the maintenance of proliferative and renewing progenitor stem cells. Embryonic stem cells, derived from blastocysts, can differentiate into cells of different lineage. (135) The stem cells can divide to produce daughter (progenitor) cells. (135)

On a cellular level, FOXD3 was shown to stain within the nucleus of luminal epithelial cells and the cytoplasm of stromal cells.

Figure 4.2.2

An immuno-fluorescence image to show the presence of FOXD3 within the stromal and luminal epithelial cells in a cultured normal human endometrium sample



The immuno-fluorescence image above shows the luminal epithelial cells, which is stained by the expressed cytokeratin. Surrounding this are the endometrial stromal cells. FOXD3 is shown to stain centrally (purple) in the nucleus of the luminal epithelial cell.

4.2.3- FOXD3 Expression in the eutopic endometrium of women with endometriosis

We found the expression of FOXD3 to be proportionately higher in the glands and luminal epithelium of endometrium in women with endometriosis compared to normal fertile control patients. In contrast to the change seen of

FOXD3 in the normal endometrium, we found there to be an increase in the expression of FOXD3 from the proliferative phase to the secretory phase within endometriosis.

The expression of FOXD3 in the endometrial stroma in endometriosis patients in the proliferative phase was weak, however increasing Slightly in the secretory phase.

There is a very strong expression of FOXD3 in the luminal epithelium and glands in the endometrium of endometriosis patients in the secretory phase. The expression of FOXD3 in these compartments during the secretory phase is higher than the FOXD3 expression in the normal endometrium during the proliferative phase. Thus, these findings indicate that there is a greater expression of FOXD3 in the glandular areas and luminal epithelium in the endometrium of women with endometriosis compared to normal control patients.

We can suggest some possibilities into why there is a higher expression of FOXD3 in patients with endometriosis. FOXD3 has a greater role in patients with endometriosis due to the increased development of stem/progenitor cells.

The endometrial lining of the uterus displays functions of stem cell regeneration.

A study by Leyendecker et al in 2009 compared the expression pattern of progesterone receptors, oestrogen receptors and P450 aromatase in the normal endometrium to ectopic endometrial implants. (136) It was found that the basalis layer of the endometrium, which possessed endometrial stem/progenitor cells, was shed in greater quantities in the menstrual flow in endometriosis patients. (136) This showed endometrial implants occur due to retrograde menstruation of endometrial stem/progenitor cells. (136) Bone-marrow derived stem cells targeting the uterus may differentiate into functional endometrium. This observation may be responsible for supporting

the coelomic metaplasia theory explained in Chapter 1, in which extrauterine stem/progenitor cells can travel to ectopic sites through the lymphovascular spaces. (136)

With Leyendecker et al suggesting more basalis layer is shed in the endometrium of patients with endometriosis, in response; there will be a greater production and regeneration of the lost stem cells. Sampsons theory proposes these shed endometrial cells will be implanted ectopically. These stem cells continually divide and grow to form progenitor cells. The increased activity of these embryonic stem cells consequently increases the expression of FOXD3.

4.2.4- The Functions of FOXD3

We can predict FOXD3 works with other expressed genes/proteins, binding to specific DNA sequences to act as a transcription factor. OCT-4 (also known as POU5F1) is also highly expressed in the embryonic stem cells. (137) Oct-4 maintains the undifferentiated state of embryonic stem cells. (137)

POU5F1 (Oct-4) decreases the activation of transcription factor FOXA1 and FOXA2, whilst FOXD3 increases the activation of them when it is expressed. The negative feedback involving these transcription factors contributes to the functioning of FOXD3 in its regulation of genes/proteins. We can suggest the repression of the transcriptional activation of FOXA1 and FOXA2 by Oct-4, is benefitting to FOXD3 functioning as a transcription factor. (137)

FOXA1 and FOXA2 bind to the DNA with the sequence 5'-[AC]A[AT]T[AG]TT[GT][AG][CT]T[CT]-3' and are involved in the regulation of apoptosis through inhibiting the expression of BCL2. (137) In referring back to Table 4.44, we discovered FOXD3 to activate regulation of BCL-2, which was seen in figure 3.32, to be up regulated during proliferative activity during endometriosis.

We suggest through the shedding of endometrial cells, as well as the migration, implantation and adhesion of ectopic endometrial cells, embryonic stems cells in these ectopic implants will produce FOXD3 and Oct-4.

This process of repair and regeneration of tissue promotes the recruitment of immune-related factors such as SPP1, CXCR4 and those mentioned earlier. We propose that the expression of the listed genes/proteins in table 3.44 during the various pathological processes (in endometriosis) may be dependent on the production of FOXD3 by embryonic stem cells.

4.3- IHC of AR/PR

To further validate our bioinformatics findings we also conducted an IHC examination of the same endometrial samples for the expression of androgen and progesterone receptors. Both receptors have already been implicated by other authors to be involved in the pathology of endometriosis as well as being highlighted in playing a role in the pathogenesis from our bioinformatics investigations.

However, our IHC study did not show a statistically significant difference in the staining of progesterone and androgen receptors in the endometrial samples from women with endometriosis.

4.3- Discussion of Androgen Receptor (AR) expression

There is available evidence to show the change in expression of AR in the normal endometrium across the menstrual cycle. Horie et al in 1992 examined 14 women with normal menstrual cycle for the expression of AR in the endometrium. There was seen to be a high expression of AR in the endometrium in both the proliferative and secretory phase in the normal endometrium. (33) There was a higher expression of AR in the stromal cells and glands of the functional layer compared to those in the basal layer. (138)

Mertens et al in 1996 however showed a greater contrast in the expression of AR in the endometrium over the two cycles. (139) It was found that AR expression was higher in the proliferative phase than in the secretory phase. (139) Mertens et al also discovered the expression of AR was higher in the stromal cells of the endometrium in proliferative phase. (139) The expression of AR in the epithelial cells was seen to be highest in the secretory phase. (139)

There is also evidence suggesting varied expression of AR in the endometrium of endometriosis patients. Carneiro et al in 2008 found an increase in the expression of AR in glandular and endometrial stromal cells in the eutopic and ectopic endometrium. (140) This increased expression of AR in the endometrium suggests a role in the pathogensis of AR in endometriosis. The study indicates androgens may be formed within endometriotic tissue and that local and systemic androgens can act on endometriotic cells. This gives a plausible explanation into why danazol (as explained in Chapter 1: Introduction) may inhibit endometrial cell growth.

Our findings showed a slight decrease in the expression of AR in the glands of the functional layer from the proliferative phase to the secretory phase. The expression of AR in the luminal epithelium decreased as well from the proliferative phase to the secretory phase in the normal endometrium. This finding differs to that found in Mertens et al, which states AR expression in the luminal epithelium is highest in the secretory phase. Our study found AR expression in the stroma to be very high in the proliferative phase, decreasing slightly in the secretory phase. This is similar to that of Mertens et al, which stated AR stromal expression is higher in the proliferative phase in the normal endometrium. The expression in the basal glands is also low in the normal endometrium, which correlates to his findings.

Our findings displayed that there was no change in expression of AR in the endometrial stroma in the endometrium of patients with endometriosis. There was no change in the expression of AR between the proliferative and secretory phase. There was also no change seen in the expression of AR in the basal or functional layer glands between patients with endometriosis and

the normal endometrium. Therefore our findings differed greatly to other published studies such as those found by Carneiro et al in 2008.

4.4- Discussion of Progesterone receptor (PR) expression

There is available evidence, which suggests that there is PR expression in the normal endometrium.
Jones et al in 1995 discovered that progesterone receptor expression in the luminal epithelial and glandular cells decreased significantly between the proliferative and secretory phases in the normal eutopic endometrium. (22) PR expression in the eutopic endometrium of endometriosis patients was found not to differ from the control endometrium. (141) Also, stromal progesterone expression was reduced in the ectopic endometrium of endometriosis patients during the cycle. (141)

Nisolle et al in 1994 showed that the highest concentration of PR occurred in the epithelial and stromal in the late proliferative phase of the menstrual cycle and decreased greatly. (142) Nisolle et al stated that in peritoneal endometriotic lesions, the highest concentrations of PR were found during the late proliferative phase in the epithelial and stromal cells. (142)

PR is usually expressed as two isoforms, PR-A and PR-B, arising from the same gene. (144) The two receptors are identical in the DNA binding domain (DBD) and C- terminal ligand binding domain (LBD). (144) There is seen to be functional differences in both receptors with PR-A possessing more importance in the uterus and ovary. (144)

Fazleabas et al in 2010 developed the baboon model of induced endometriosis, explaining the retrograde menstruation hypothesis. The study suggested there was a progesterone resistance and decreased responsiveness of the progesterone receptor (PR) in patients. (143) The study notes progesterone resistance to be a gradual process, which may become evident after 6 months of having the disease. (143)
Attia et al found PR-B to be present in 17 out of 18 eutopic endometrial

samples, and found its level to be increased in the pre-ovulatory phase. (144) PR-A was detected in all samples but in lower levels than PR-B. (144)

There was no change seen in the expression of PR in the glands of the normal endometrium from the proliferative to the secretory phase. Similarly, in the stroma of the normal endometrium, there was seen to be no clear cyclic change in the expression of PR. These findings differ to those already published on the changes of PR expression in the normal endometrium.

The expression of the luminal epithelium slightly decreases from the proliferative to secretory phases. Similarly, this finding differs to Nisolle et al's study, which states the change is quite predominant.

Our experimental findings showed that there was no cyclic change (increase or decrease) in the expression of PR in the epithelium and the basal layer glands in the endometrium between patients with and without endometriosis. We also found the change in PR expression in the stromal and functional layer glands in the endometrium in endometriosis to be statistically insignificant.

There are many conflicting studies that provide differing ideas into how PR expression may or may not change during endometriosis. Therefore, our findings agree with some ideas suggested by Jones et al in 1995 however conflicting the proposals stated by Fazleabas et al in 2010.

The genes/proteins up regulated within the pathological events during endometriosis explained in previous chapters were controlled by transcription factor FOXD3. We have shown that FOXD3 is expressed in the human endometrium and aberrantly expressed in the endometrium of women with endometriosis.

4.5- Deficiencies of the study

There were a few deficiencies and flaws within our study. We feel the following issues may have contributed to our study obtaining insignificant data when investigating the AR and PR staining in endometriosis, possibly hindering us from obtaining a true result.

The first deficiency was the small sample size. We only used 20 endometrial samples (n=20), 10 endometriosis samples and 10 normal fertile control endometrial samples. With not enough available collected samples from surgery, a small sample size may have provided a lack of variety in our data as well as questionable validity and reliability in our findings. The availability of samples was dependent on suitable surgeries occurring during the week.

We were also limited in using IHC (Immunohistochemistry) in our laboratory work when validating our bioinformatics results. This is a form of qualitative analysis which may be more subjective (object of visually scoring slides may be scored differently between other assessors) than quantitative forms of analysis such as Reverse Transcription Polymerase Chain Reaction (PCR) or Western Blotting.

We also did not use ectopic lesion samples out of the 10-endometriosis samples we had. We only used full thickness and pipelle samples from the eutopic endometrium. Studies have proposed the structure and function of endometrial ectopic lesions may differ from that of the eutopic endometrium in patients with endometriosis.

And finally, we failed to conduct any in-vitro functional work on our desired transcription factor or any of the genes we found to be up regulated within endometriosis. We therefore were unable to discover experimentally if FOXD3 or any of the up-regulated genes obtained from our bioinformatics findings had an effect on the endometrial function in cultured cells.

Considering all these deficiencies, we have proposed potential experimental improvements as well as possible future work. This would look to build upon the findings we have obtained, and launch a further study into how FOXD3 and genes/protein we found to be up regulated could play a role in the

pathogenesis of endometriosis as well as possibly involvement in a target for therapeutic or treatment purposes.

4.6- Potential experiment improvements and Future work

4.6.1-How can the Bioinformatics study be extended?

In conclusion, I felt we conducted a comprehensive bioinformatics approach on the collated data we inputted. In learning and understanding all of the relevant software before we used it, we were able to appreciate the relevance of the data and analysis produced. However improvements could have been made.

There could have been a greater use of sophisticated network analysis tools after obtaining the networks of genes/proteins during Ingenuity. These network analysis tools would be used to separate the protein-interaction and gene-interaction within the mapping system.

Analysis tools may have been used to perform an analysis of the topology of the resultant network finding the hub and shortest path in the network. This would all contribute to an increased understanding of pathological pathway processes during endometriosis and a more specific interaction of the genes/proteins involved.

Cytoscape, which has numerous useful analytical plugins could have been used to analyze the inputted data. Like Ingenuity, Cytoscape provides a powerful visual mapping system across networks of data, as well as molecular and genetic interaction data sets in many formats.

String software could have been used to compliment our findings. String is a software program that is able to integrate interaction data from a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations and are derived from genomic context, highly throughput experiments and conserved co-expression. This software program is able to use other species information

through orthology to human proteins and this may help obtain clues that were missed due to an absence of information for humans.
Overall, a fundamental improvement would be to achieve further experiment validation of our bioinformatics analysis. Upon achieving this, we can return to input these findings back into the computational technology system networks.

4.6.2-How can the IHC study be improved?

We can propose improvements for any future study relating to FOXD3 and endometriosis. Improved experimental work can be combined with a stronger bioinformatics approach to obtain more insightful findings in the future with the purpose of gaining further understanding into the pathogenesis of endometriosis. Future work can also use our bioinformatics and experimental findings to investigate potential improvements in therapeutic and treatment targets.

The sample size would need to be increased from 20 endometrial samples to a much larger number. This sample size increase would provide more strength to our findings. With a larger sample size, more accurate quantitative statistical analysis can be achieved on the larger dataset. Additionally, with a larger endometrial sample size, there would be a more diverse set of endometriosis samples at different stages of the disease. Therefore, with a larger sample of patients with endometriosis at different stages, genetic and molecular changes could be grouped to the different stages of the disease and a more detailed study can be made into the pathogenesis.

It is also important in future work on FOXD3 that samples from ectopic endometrial lesions are used. Many studies have outlined functional differences in the eutopic endometrium compared ectopic lesions found in patients with endometriosis. Ota et al in 2000 through immunohistochemistry, found there to be COX-2 staining in the endometriotic tissue implanted on the ovaries. (145) The study reported that there had been larger amounts of prostaglandin (of which is controlled by COX-2) produced in ectopic endometrial tissue in endometriosis. (145) As understood from our study,

FOXD3 is a transcription factor for the enzyme PTGS2 (also known as COX-2). Hapangama et al in 2010 investigated how ectopic endometriotic lesions have excess proliferative potential in the baboon model, and in the eutopic endometrium the changes were induced by the development of endometriosis. (146)

Therefore further work of FOXD3 would benefit from investigating its expression in both the eutopic and ectopic endometrium of endometriosis samples.

4.6.3- Alternative experimental methods

Alternative methods to validate our IHC data would also be important to further confirm our findings in the pathogenesis of endometriosis. Western Blotting is an analytical technique used to detect specific proteins in a sample through gel electrophoresis, separating native and denatured proteins. A specific antibody would then be used as a probe towards this target protein (FOXD3).

Reverse Transcription Polymerase Chain Reaction (PCR) is a technique used to generate many copies of a DNA sequence after it has been reversely transcribed into its DNA complement from the RNA stand. The complementary DNA (cDNA) is then amplified using standard PCR.

Both methods would be used to conduct In-Vitro work on endometrial cells in culture, to view if FOXD3 has an effect on the endometrial cell function. IHC was able to show the increased expression of FOXD3 in patients with endometriosis and at certain times in the cycle in the normal endometrium. However, in-vitro work on live FOXD3 cultured cells would take the investigation a step further and study the exact effect of this transcription factor on the structure and function of endometrial cells, especially in patients with endometriosis.

Also, the expression and effects of FOXD3 in endometriosis could also be tested on animal models. Many studies, such as that of Hapangama et al in 2010 and others, have obtained conclusive and successful findings from

studying the aberrant expression of certain proteins in the eutopic and ectopic endometrium in a baboon endometriosis model. (38) Other studies such as that of Wilkosz et al in 2011 used enhanced green fluorescent protein in mice with endometriosis to investigate cell attachment, invasion and vascular in-growth. (147) Therefore, further experimental work with animal models will also provide further confirmation to any bioinformatics findings.

4.6.4-Study of other transcription factors identified in the bioinformatics approach

Cepba (C/enhanced binding protein) and HLF (Hepatic Leukaemia Factor) were both other notable transcription factors identified from Opossum to regulate the large set of up regulated genes/proteins during endometriosis and the related pathological processes. These can be investigated in future work, studying their relation to endometriosis and the expression with the endometrium. With an existing study investigating the regulation of p450 aromatase in endometriotic and endometrial stromal cells by Cepba, investigations could be formulated to discover if there is any the involvement experimentally of HLF in endometrial and endometriotic cells.

4.7- Final Discussion

In regards to future studies, we propose bioinformatics to systematically collate available evidence and predict the involvement of further key players. The combination of a bioinformatics approach and laboratory studies would make future studies in the pathogenesis of endometriosis and other diseases more effective and efficient in providing an understanding for the wider public and those affected.

Our bioinformatics work was able to show how certain genes were up regulated in common pathological processes during endometriosis, and how these genes and processes were interlinked. The software programs such as

Ingenuity and Genevestigator were able to show the expressional pattern of the genes in the pathological processes within endometriosis and compare the up-regulation of these genes to similar expressional patterns in conditions such as gastric cancer. The identification of the common regulatory transcription factor FOXD3 through Opossum provided a breakthrough in pinpointing a key player involved in the gene regulation.

Through findings from experimental work, FOXD3 was expressed in the glandular and luminal epithelial cells of the proliferative endometrium. In endometriosis, there was an increase in the expression of FOXD3 in the glandular and luminal epithelial cells of the secretory endometrium. The findings in our thesis support the idea of the increase in expression of FOXD3 corresponding to the up regulation of the important and well known genes the factor regulates in the nucleus of the glandular and luminal epithelial cells during transcription. We were therefore able to show how FOXD3 is a key player in the pathogenesis of endometriosis through its aberrant expression in patients with the disease.

As mentioned earlier, extensive future work is needed for FOXD3 and its potential effects on the function of the endometrium in patients with endometriosis. With an aim of discovering future details regarding the pathogenesis of endometriosis, future work involving FOXD3 and a bioinformatics approach is important in studying how the transcription factor may be targeted for therapeutic use. Potential future discoveries can be integrated into clinical settings, being used during treatment targets, therefore providing a greater advance in the progression of combating endometriosis.

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