The Emerging Role of Epigenetics in the Aetiology of Endometriosis

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Abstract

BACKGROUND. Endometriosis is a very common but poorly understood disease. Defined as the presence of tissue resembling endometrial glands and stroma found in various locations around the body the disease manifests as implants which periodically proliferate and bleed. Our understanding of the mechanisms underlying the disease is limited. No methods are available for predicting the incidence of endometriosis around the world. There are many theories concerning the origin of endometriosis that contradict and complement each other. Heritable changes in gene expression independent of changes to DNA sequence, often referred to as epigenetics, is being considered as one possible mechanism via which endometriosis can develop. Epigenetics is a relatively new field of research that has revolutionised the understanding of complex diseases such as cancer and diabetes.

AIMS AND OBJECTIVES. Firstly, this thesis aims to provide a novel model for predicting the incidence of endometriosis based on known epidemiological risk factors. The second aim of this thesis is to investigate the involvement of epigenetic mechanisms such as DNA methylation, loss of imprinting and aberrant microRNA expression in the aetiology of endometriosis.

METHODS. For the prediction of endometriosis, data on known epidemiological risk factors for endometriosis, including diet and parity, were collected for 121 countries from various international databases such as the Food and Agriculture Organisation and World Health Organisation, normalised and used to generate a model predicting patterns of endometriosis risk. For the epigenetic study, systematic reviews and critical analysis was carried out on the literature concerning DNA methylation and transgenerational epigenetic inheritance, as well as other epigenetic mechanisms found to be disrupted in endometriosis. Bioinformatic analysis of imprinted gene databases and microRNA prediction software was cross referenced with existing microarray data on endometriosis to identify novel imprinted genes and microRNAs that may be involved in the aetiology of endometriosis.

RESULTS AND DISCUSSION. The prediction model reveals that the incidence of endometriosis is most prevalent in Northern Europe and North America, with the lowest incidences in equatorial Africa. Asia and South America appear to be areas of intermediate risk. The incidence of endometriosis, predicted from the proposed model, concurs with what limited knowledge currently exists concerning the incidence of endometriosis, based on previous epidemiological studies, suggesting that it is reliable. This preliminary model provides, for the first time, information about endometriosis prevalence outside Europe, America and Australia and presents a basis for a more detailed epidemiological investigation into endometriosis. Analysis of epigenetic data identified the

aromatase cycle and estrogen sensitivity in endometriotic cells as having an epigenetic origin. Two imprinted genes, ABS4 and BEGAIN, were identified as being disrupted in endometriosis, with the potential for epigenetic disruption of IGF-2 in endometriosis also discussed. Numerous microRNAs, such as mir-23a and mir-29a, were identified as playing a significant role in the immune system dysfunction observed in endometriosis. The evidence for environmentally induced endometriosis being transmitted to future generations by epigenetic means is also presented.

CONCLUSION. A promising new approach for predicting the incidence of endometriosis has been developed that can be further developed in the future to improve its predictive accuracy and robustness. The combined bioinformatics and genomic data analysis identified aberrations in imprinted genes and microRNAs that may play a significant role in the development of endometriosis. This provides the basis for future studies exploring these systems that may provide novel therapeutic strategies for the treatment of endometriosis.

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Abbreviations

17β-HSD1	17-beta-hydroxysteroid dehydrogenase 1
AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
COUP-TF	Chicken ovalbumin upstream promoter – transcription factor
COX-2	Cyclo-oxygenase-2
CpG	Cytosine – Guanine pair
CYP	Cytochrome P450
DES	Diethylstilbestrol
DNA	Deoxyribosenucleic acid
DNMT	DNA Methyltransferase
ER	Estrogen Receptor
ESC	Endometrial Stromal cells
FAO	Food and Agriculture Organisation
HDI	Human Development Index
HOX	Homeobox gene
ICR	Imprinting control region
IFN-γ	Interferon gamma
IGF	Insulin growth factor
IL	Interleukin
LOH	Loss of Heterozygosity
LOI	Loss of Imprinting
miRNA	Micro RNA
mRNA	Messenger RNA
NF-κB	Nuclear factor – kappa B
NK	Natural killer cell
NMDA	N-methyl-D-aspartic acid
PGE_2	Prostaglandin E2
PM	Peritoneal macrophage
PR	Progesterone Receptor
RANTES	Regulated up activation T cell expressed and secreted
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
SF-1	Steriodogenic factor-1
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF-β	Transforming growth factor beta
Th1	T helper type 1
Th2	T helper type 2
TNF-α	Tumour necrosis factor alpha
tRNA	Transfer RNA
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Chapter 1: Introduction

Endometriosis is a complex but very common gynaecological disease, often described as enigmatic due an incomplete understanding of the exact aetiology of the disease. Endometriosis is defined as the presence of endometrial glandular and stromal cells in extra uterine locations. These endometrial cells invade and proliferate forming endometriotic implants. These implants behave like normal (eutopic) endometrium, i.e. they proliferate and bleed in conjunction with hormonal cycles. The activity of these implants can lead to painful internal scarring, dysfunction of the affected organ/s and the formation of inter-organ adhesions.

Endometriosis is most commonly characterised by numerous painful symptoms brought on by the activity of the endometriotic implants but is also associated with numerous other symptoms that can lower the quality of life of the sufferer. The most common symptoms include dysmenorrhoea (painful periods), dyspareunia (painful intercourse), chronic pelvic pain (defined as more than 6 months of non cyclic pain), abnormal uterine bleeding, and reduced fertility. Less common symptoms include chronic fatigue, painful bowel movements (dyschezia), painful urination (dysuria) or pain in areas other than the pelvis, such as back, legs or shoulders. There are also numerous psychological symptoms, such as depression, that can be associated with endometriosis due to the heavy emotional burden of the disease.

Endometriosis is a common disease, it is approximated that endometriosis affects 1 in 10 women of reproductive age [1], although estimates range from 5% to 20% of the general population [1]. There is some evidence to suggest endometriosis is more common in non-Hispanic Caucasians than women of African descent [2, 3], however studies on the racial differences of endometriosis are very few in number. Although endometriosis was previously considered to be a disease that affected women approaching middle age, it is now recognised that the disease is diagnosed more frequently

in younger women [4]. The symptoms of endometriosis can present as early as the onset of puberty, particularly around the time of menarche. However, as patients find it difficult to get their symptoms taken seriously and misdiagnosis is common, there are extensive delays in the diagnostic process, meaning that the average time for a woman to be diagnosed with endometriosis can be up to 9 years. There are numerous factors compounding the diagnostic process, foremost of which is the heterogeneous nature of its symptoms that can often lead to misdiagnosis. A survey of women from the United Kingdom found that 68% of women were initially misdiagnosed, most commonly with irritable bowel syndrome [5]. Furthermore, accurate diagnosis can only be achieved via a laparoscopy, whereby a camera is inserted into the pelvic cavity for visual assessment of any endometriotic implants [6]. The accuracy of this procedure is dependent on factors including the quality of the equipment and the skill of the surgeon. This may be the reason that the incidence rates of endometriosis are only known for a handful of Western countries, with data on incidence from other countries simply non-existent.

Endometriosis is not only a heavy burden for the sufferer, but also in economic terms. The loss of work hours caused by illness due to endometriosis is estimated to cost the UK economy over £2billion a year [7]. There is also the cost to the National Health Service to take into consideration in terms of diagnostic/therapeutic surgeries and medical treatments which may add further billions to the total economic cost of endometriosis. A recent study from the USA, for example, found that the average cost of an individual surgical procedure ranged from $4,289 to $11,397 depending on its nature [8]. Given that many women with endometriosis require multiple surgical procedures it is likely endometriosis is extremely costly in economic terms.

Despite increased research into the origin and pathophysiology of endometriosis there are still many gaps in our knowledge that needs to be filled. It is therefore imperative to implement new and emerging scientific concepts into our understanding of endometriosis.

1.1 On the Origin of Endometriosis

How endometriosis arises within the body has been a matter of debate ever since the disease was first described over 300 years ago as 'cysts' on the organs of the pelvic cavity, and later described in detail in 1860 by a physician named Von Rokitansky [9]. However, it wasn't until 1927 when a physician from Albany, New York named John Sampson began formulating his theories on the origin of endometriosis that our understanding of the disease began to flourish [10]. Sampson's pioneering work provided the basis for the theory of retrograde menstruation for the origin of endometriosis, which is still the most widely accepted theory on how endometriosis arises.

1.1.1 Retrograde Menstruation

Sampson postulated that during normal menstruation, menstrual debris including viable eutopic endometrial cells, cytokines and growth factors could travel in a retrograde fashion through the fallopian tubes and into the pelvic cavity [10], here these cells could invade and proliferate on surrounding tissues (Figure 1). This theory is supported by the histological similarity between ectopic (endometriotic) tissue and normal (eutopic) endometrial tissue (Figure 2) and the frequent occurrence of endometriosis on the organs of the pelvic cavity, such as the uterus, uterosacaral ligaments and the ovaries [11]. Additional support for this theory was provided by the finding that retrograde menstruation occurs in 90% of menstruating women [12]. Furthermore, forced surgical induction of retrograde menstruation in primate models leads to the development of endometriosis in 50% of cases [13]. However, there are drawbacks to Sampson's theory. For example, retrograde menstruation cannot account for the occurrence of endometriosis in extra pelvic locations [14]. Additionally, endometriosis is estimated to affect 1 in 10 women of reproductive age, equating to 2.2 million sufferers in the U.K alone. Yet, the occurrence of retrograde menstruation is near universal, therefore the question remains as to why only a fraction of the population develops endometriosis?

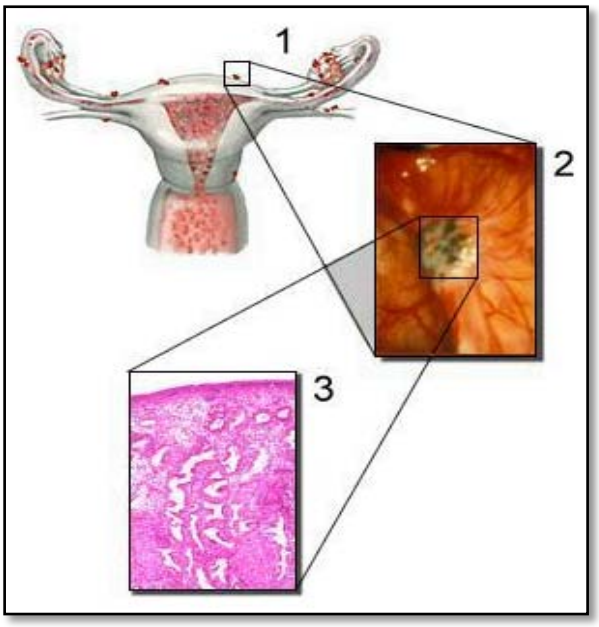


Figure 1. Retrograde Menstruation

Illustrating endometrial cells travelling through the fallopian tubes onto the surface of the pelvic organs where they implant and proliferate (1). Here they form endometriotic implant of varying severity (2) which contain glands and stroma normally found in the eutopic endometrium (3).
(Images courtesy of Dan Martin; The Fertility Institute of the mid-South, 2000-2007. AstraZeneca, 2008 and Cornell University Medical College, 1995.

A further confounding issue for the retrograde menstruation theory is the observation of endometriotic implants in men undergoing estrogen therapy for prostate cancer to consider [15-18]. It is well documented that the main trophic factor in endometriosis is estrogen [19], therefore it may well be possible that estrogen exposure plays a significant role in development of the disease.

Figure 2. Histology of Endometriosis

The left hand image shows normal endometrium with glands and stroma the dominant cell types. In the right hand image similar endometrial glands and stroma can be observed (arrows) only these are embedded deep into the wall of the bowel, illustrating a classic case of colonic endometriosis.
Images courtesy of Wolters-Kluwer, 2009 and University of Utah, Health Sciences Centre, 1993-2009

1.1.2 Coelomic Metaplasia

In response to the limitations of retrograde menstruation, Gruenwald proposed in 1942 [20], that mesothelial cells of any organ, including those of the pelvic cavity, in particular the ovary, could undergo differentiation into functional endometrium; this came to be known as the theory of coelomic metaplasia and has since gathered support from several authors [21-24]. The process by which cellular differentiation into functional endometrium occurs remains speculative. It may be that steroid hormones or exogenous compounds induce the differentiation of normal mesothelial cells into endometriotic cells. Validation of this theory rests on its ability to explain how amenorrheic women and men undergoing hormone therapy occasionally present with endometriosis [15]. Additional support for this theory is the prediction that endometriosis may be found anywhere where mesothelium is present. Findings of endometriosis in the pleural cavity [25], diaphragm [26], brain [27] and several other organs [28-30] therefore adds credibility to the theory of coelomic metaplasia. However, it must be noted that metastasis of endometrial cells through the circulatory or lymphatic system may also account for the presence of endometriosis at these extra-pelvic sites [31].

1.1.3 Immune System Abnormalities

It has long been recognised that patients with endometriosis display alterations in the immunologic response. It is thought that defective immunosurveillance [32] may decrease the clearance of any refluxed menstrual debris, allowing the persistence of ectopic endometrial cells within the pelvic cavity. Additionally, it is also thought that the observed abnormal immune response could promote the persistence and growth of ectopic endometrial cells [32, 33]. In the peritoneal fluid (PF) of women with endometriosis an increased concentration of macrophages has been reported [34]. Macrophages are involved in recognising foreign and damaged cells in the peritoneal cavity, once recognised these cells are then processed by macrophages for presentation to T lymphocytes. However, in patients with endometriosis malfunction of the peritoneal macrophages (PM) induces

them to secrete growth factors and cytokines that may promote the survival of ectopic endometrial cells. Similarly an alteration of the production of cytokines from T helper lymphocytes may induce changes in the consistency of the PF allowing a favourable environment for ectopic endometrial tissue proliferation. The regulation and activation of macrophages and lymphocytes is dependent on a fine balance of cytokine expression a balance that is upset in endometriosis [35]. A summary of the cytokines and growth factors thought to be involved in the pathogenesis of endometriosis is shown in Table 1.

Table 1. Immunologic factors involved in the pathogenesis of endometriosis

Factor	Secreted from	Function
IL-1	PM, ESC	May promote the survival of endometriotic cells by altering secretion of other cytokines such as IL-6, IL-8 and TNF-α [36, 37]
IL-6	PM, ESC	Promotes differentiation of macrophages and secretion of other cytokines
IL-8	PM	A potent angiogenic agent, thought to promote the neovascularisation of endometriotic implants [38]
TNF-α	PM	Levels of TNF-α correlate strongly with disease stage [39]. May promote adherence of ectopic cells [40]
IL-4	Th2	Inhibits production of IL-1, IL-6 and TNF-α. Stimulates production of IL-8 [41]
IL-10	Th2	Involved in suppressing the immune response [42]
TGF-β	PM	May act as a mitotic agent for ectopic endometrial cells [43]
RANTES	ESC	Involved in recruiting leukocytes [32]
IFN-γ	Th1	Thought to be embryotoxic, activates macrophages [44]

IL = Interleukin, TNF-α = Tumour Necrosis Factor α, TGF-β = Transforming Growth Factor β, RANTES = Regulated upon Activation Normal T cell Expressed and Secreted, IFN-γ = Interferon-γ, PM = Peritoneal Macrophage, ESC = Endometrial Stromal Cell, Th1 = T-helper 1 cell, Th2 = T-helper 2 cell.

Table 1 is by no means a definitive list of all the immune system factors involved in endometriosis, rather it is a shortlist of the key components. The altered activity of these key components, many of which are involved in the inflammation response, also illustrates why endometriosis is considered a chronic inflammatory disease. The activation of inflammatory pathways is also a possible cause for

the pain associated with endometriosis [45]. However, it is not just *over* activity of the immune system that contributes to the pathogenesis of endometriosis. Decreased activity of natural killer (NK) cells has also been implicated in the pathogenesis of endometriosis [46, 47].

NK cells are responsible for clearing tumour cells, foreign cells and virus infected cells from the body and are activated by cytokines such as IL1, IL-2, IL-12, IFN-γ and TNF-a [48, 49]. Thus, it would be expected that NK cell activity should be up regulated in patients with endometriosis, however extensive experimental data shows that this is not the case [46, 47, 50, 51]. There have been several attempts to reconcile this paradox. It has been suggested that stimulation of antibodies by the presence of ectopic endometrium could lead to the formation of complexes that bind to Fc receptors present on the surface of NK cells that inhibit their function. Additionally, the production of prostaglandin E (PGE_2) by activated macrophages may suppress NK activity [49, 52].

1.1.4 The Genetics of Endometriosis

Endometriosis is recognised as a heritable disease due to the finding that endometriosis is significantly more common in first degree relatives of women with the disease [53-55]. Additionally, a study of monozygotic twins reported that endometriosis was concurrent in 14 out of 16 twin sets [56], with further twin studies confirming the likelihood of endometriosis arising more frequently in close relatives [57, 58]. The heritable nature of endometriosis has prompted many to investigate which gene or sets of genes are responsible for the disease. To date many deregulated genes have been identified in endometriotic cells [59-62] with a wide variety of functions including apoptosis, cell cycle regulation, vascularisation, immune system regulation and cell adhesion. However, identifying the exact gene/s responsible has thus far failed. Some authors have linked certain genetic polymorphisms to endometriosis [63], however efforts to identify genetic polymorphisms consistent across cultural and ethnic backgrounds have also failed [63]. A multi-centre study between England and Australia which examined any regions of chromosomal linkage in sister paired endometriosis

sufferers has however narrowed down the region of interest. The results of this study identified that chromosome region 10q26 is significantly associated with endometriosis [64] and more recent studies have indicated region 7p15.2 is significantly associated with endometriosis [65], however no individual genes have been identified that may explain the heritable nature of endometriosis.

More modern thinking has led to implications for the relatively new field of epigenetics in the origin and development of endometriosis. Epigenetics concerns heritable changes to gene expression that can be influenced by environmental factors but are not the result of changes to the DNA code. Examples of epigenetic mechanisms include; DNA methylation, loss of imprinting and gene regulation by microRNAs. Epigenetics has revolutionised the understanding of other complex multifactoral diseases such as cancer [66-68]. Recently it has emerged that epigenetic mechanisms likely play a significant role in the origin and progression of endometriosis [69]. Therefore, the application of an understanding of epigenetic mechanisms to the pathophysiology of endometriosis may lead to a better understanding of the origin and aetiology of the disease. Further discussion of epigenetics and its role in endometriosis is provided in Chapter 3.

1.1.5 Environmental/Lifestyle Factors and Endometriosis

Epidemiological data regarding endometriosis is currently limited. The few studies that have been undertaken suggest that lifestyle and dietary factors may be associated with susceptibility to developing endometriosis [70-72]. The results of these epidemiological studies found that a diet high in fruit and vegetables and low in meat products was protective against developing endometriosis. Additionally, women with few or no children and low body mass index (BMI) were at a higher risk of developing endometriosis. A summary of the lifestyle and environmental factors thought to be involved in the susceptibility to endometriosis are summarised on Table 2.

Table 2. Environmental and lifestyle factors associated with endometriosis

Risk Factor	Increase/Decrease Risk
Meat Consumption	↑
Alcohol Consumption	↑
Low BMI	↑
Fruit and Vegetable Consumption	↓
Parity	↓
Smoking	↓

Other authors have suggested that exposure to synthetic compounds such as dioxin and other polychlorinated biphenyls (PCBs) could lead to the development of endometriosis due to their effects as endocrine disruptors [73, 74]. Dioxin is a by product of the chlorine bleaching process used in the wood pulp processing industry, this also includes the manufacture of tampons which is thought to be a major source of dioxin exposure in women. However, the associations with dioxin are mainly based on animal data [75-77] which some authors criticise for poor study design and data analysis [78]. Human data on dioxin exposure and endometriosis risk is scant and in some cases appear contradictory. For example, a study reported that the incidence of deeply infiltrating endometriosis in Belgium, reportedly the highest in the world, correlates with high dioxin exposure through breast milk [79]. However, another study assessed massive dioxin exposure from the Seveso incident in Italy during the summer of 1976, whereby a chemical manufacturing plant accidentally released 1Kg of dioxin into the atmosphere, showering the neighbouring residential areas with dioxin. Although extremely high levels of dioxin contamination were found in soil and water samples, no significant increase in endometriosis incidence were observed, even after a 26 year follow up study [80]. Despite reported increased serum dioxin levels [81, 82], and increased serum levels of bisphenols [83] observed in endometriosis patients, a conclusive association between environmental toxicant exposure and increased risk of developing endometriosis has yet to be established.

Given the variety and conflicting notions pertaining to the origin and development of endometriosis, it becomes clear why endometriosis is often referred to as the '*disease of theories'*.

1.2 The Structure and Function of the Endometrium

Endometriotic cells are thought to be derived from the normal endometrium, therefore an understanding of the normal function of the endometrium is essential for the understanding of endometriosis [84]. The primary functions of the endometrium are to allow the implantation of the blastocyst and provide mechanisms for the clearance of tissue and haemostasis at menstruation. In order to achieve this the endometrium undergoes dramatic changes in structure and function during each menstrual cycle, which is classified into four distinct phases: menstrual, proliferative, ovulatory and secretory (Figure 3). It is normally stated that the menstrual cycle lasts 28 days, however it can vary from 20 to 34 days. The structural and functional changes that occur during each phase are the result of changes in the molecular components of the cells within the endometrium and are under the control of ovarian (estrogen and progesterone) and pituitary (Follicle stimulating Hormone and Luteinising Hormone) hormones. The function of the endometrium is sensitive and tightly controlled, therefore abnormal or inappropriate cyclic changes are thought to be responsible for many common disorders of the female reproductive system, including abnormal uterine bleeding, infertility, endometriosis, adenomyosis and endometrial cancer [85].

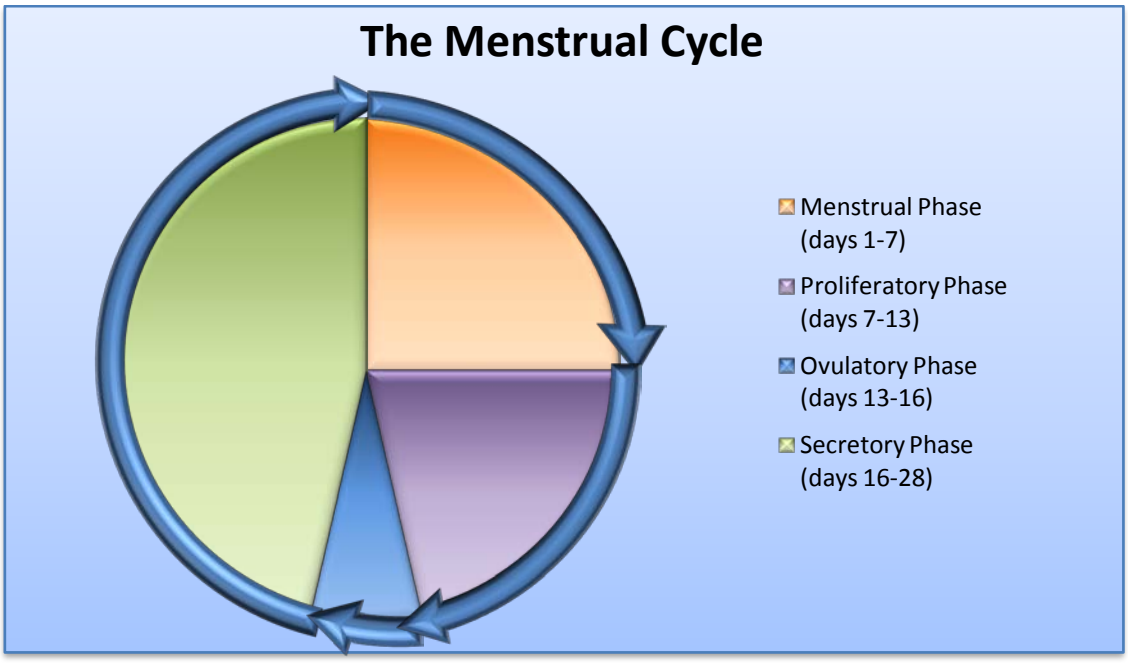


Figure 3. The menstrual cycle. Adapted from; *Endometriosis in Clinical Practice*, by David Olive (2005) Taylor and Francis

The endometrium is composed of multiple cell types that form two distinct layers, the stratum basalis (which forms a constant basal layer for endometrial regeneration) and the stratum functionalis (which forms the section of the endometrium that proliferates and is shed during the menstrual cycle). The stratum functionalis is further divided into its various cell types, the stroma, glandular and luminal epithelium (Figure 4).

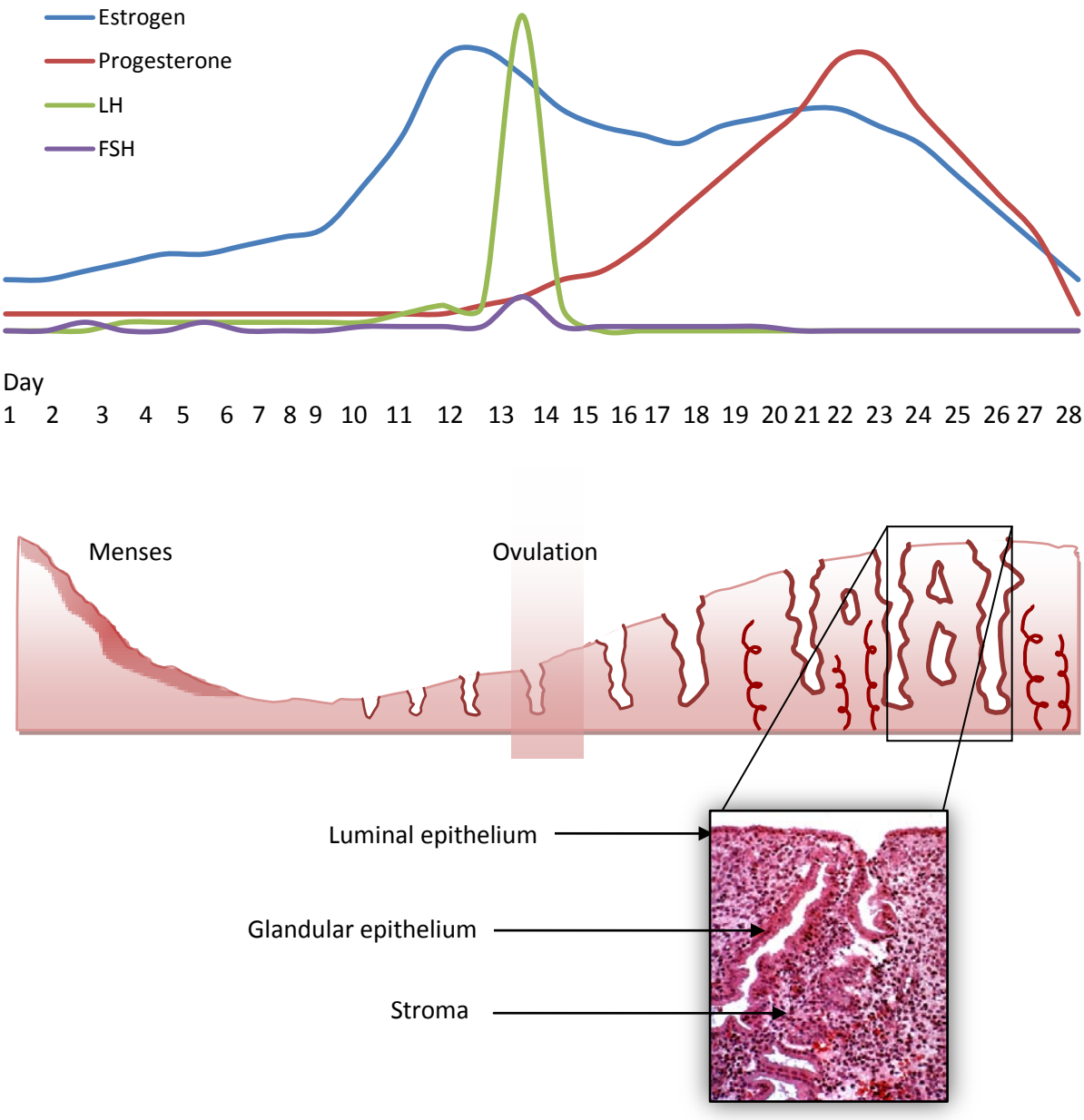


Figure 4. The structure of the endometrium during the menstrual cycle
Showing the structure of the endometrium over the menstrual cycle and a micrograph image of the cellular components of the stratum functionalis. The glandular epithelium are a secretory gland, producing factors important for embryonic implantation. Adapted from; *Endometriosis in Clinical Practice*, by David Olive (2005) Taylor and Francis. Image courtesy of Current Medical Group, 2009.

Day one is often the onset of menses, and the proliferatory phase that follows is characterised by re-epithelialisation occurring as early as day 2 [86]. By days 5-7 the endometrial epithelium covers the surface of the endometrium. A rise in estrogen levels over days 8-14 results in increased epithelial and stromal mitosis as well as proliferation of the glands. Constantly high levels of estrogen result in a surge of luteinising hormone (LH) which triggers ovulation about 34-36 hours after the LH surge. The resulting oocyte originates from an ovarian follicle which progresses to become a corpus luteum initiating the secretion of progesterone and estrogen. This signals endometrial transformation to the secretory phase. During the early secretory phase (days 15-18) progesterone levels are low and endometrial mitosis continues. During days 18-23 progesterone levels steadily rise and the endometrial glands secrete increased levels of pro-implantation factors (such as uteroglobin, trophinin and VEGF) into the lumen and arterioles also become spiral in appearance. If embryo implantation does not occur during the 'window of implantation' which is defined as days 20-24 then the late secretory phase ensues from days 24-28. During this phase falling levels of estrogen and progesterone produce marked changes in tissue structure. Stromal decidualisation occurs along with regression of glandular epithelium and by day 27 specialised uterine leukocytes infiltrate. Finally, focal necrosis and haemorrhage are observed on day 28, predisposing the onset of menstrual bleeding.

The endometrium also expresses a number of cytokines, essential for host immunity, correct cyclic timing, maintenance of pregnancy, and adhesion molecules, all requisite for successful implantation of the developing embryo [84].

Since ectopic endometrial tissue is considered to be derived from eutopic endometrium, it is reasonable to suggest that ectopic endometrial tissue responds to changes in hormonal levels in the

same way as eutopic endometrium i.e. periodic growth and shedding. As ectopic endometrial implants are located within body cavities the resultant cellular debris has no means of escape thereby accumulating within the cavity leading to internal scarring and localised chronic inflammatory responses at the site of the endometriotic implant.

1.2.1 Histology and classification of endometriosis

Endometriosis can only be definitively diagnosed via laparoscopic visualisation of the endometriotic implants. Although this is a costly and invasive procedure that carries the inherent morbidity risk of any surgery, it does allow immediate surgical intervention at diagnosis, if necessary. However, endometriosis has a wide array of appearances, meaning laparoscopy can often give false positives or negatives. Endometriotic implants can be microscopic, penetrate deeply into tissue or have a subtle appearance making them almost impossible to observe with the naked eye [87]. Efforts to develop a non-invasive test for endometriosis have met with some success. Measurement of serum levels of the cell surface antigen CA-125, a tumour marker for ovarian cancer, have shown that women with moderate to severe endometriosis have increased levels of CA-125, indicating a shared pathological characteristics between endometriosis and ovarian cancer. However, women with minimal or mild endometriosis do not have significantly different CA-125 levels than controls [88]. This is problematic as severity of disease is often unrelated to the severity of symptoms [89]. In fact it has been reported that stage of endometriosis is indirectly proportional to severity of symptoms, with women with minimal endometriosis often suffering the most severe pain symptoms [90]. Further research into proteomics found certain urinary biomarkers, such as cytokeratin-19, were notably elevated in endometriosis [91] giving further hope for a future non-invasive test. Therefore, unfortunately, there is no accurate non-invasive diagnostic test for endometriosis at present, thus diagnosis remains dependant on the visual appearance of endometriosis.

A typical endometriotic implant appears as a reddish-blue/black 'powder burn' lesion (Figure 5A), which owes its colouration to the amount of intraluminal haemosiderin and debris present [92]. A white appearance (Figure 5B) indicates a quiescent endometriotic lesion that is often difficult to distinguish between other implant types. Red endometriotic implants (Figure 5C) represent active, haemorrhagic implants with a dense vascular supply. Endometriotic implants behave much in the same way as normal endometrium in that they proliferate and haemorrhage in line with (but sometimes independently of) the menstrual cycle. This regular proliferation and haemorrhage leads to the formation of scar tissue and adhesions between pelvic organs (Figure 5D) that is thought to contribute significantly to the debilitating pain symptoms of endometriosis [93, 94].

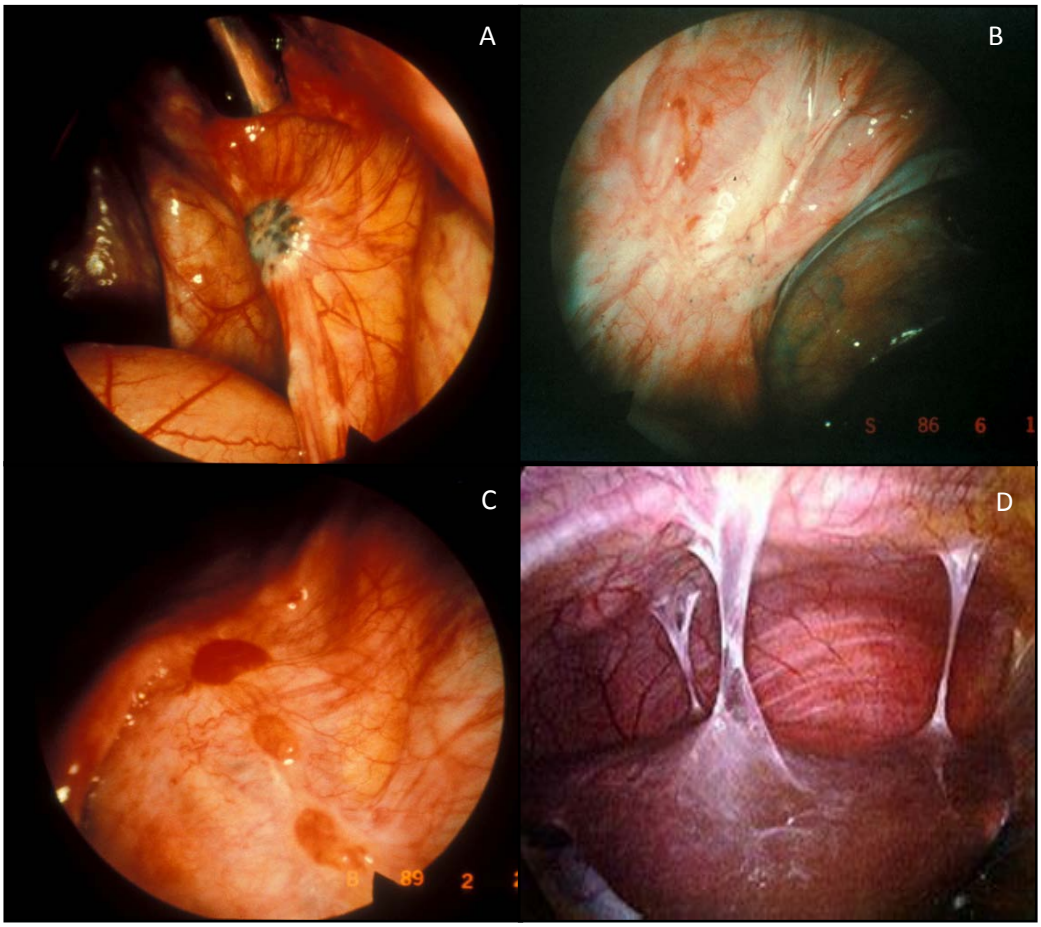


Figure 5. The various appearances of endometriosis and adhesions at laparoscopy
Images courtesy of www.endo-resolved.com, 2003-2009 and Wolters-Kluwer, 2009

Endometriosis is also classified into different stages and grades [95]. The four stages of endometriosis are minimal, mild, moderate and severe. The staging system was devised by the

American Fertility Society (AFS) which has undergone considerable updates since its inception in the 1970s. The current AFS criteria for endometriosis staging, last revised in 1996, is based on a points system. Points are assigned for the severity of endometriosis based on the size and depth of the implant and the severity of adhesions. For example 1-5 points = stage I – minimal, 6-15 points = stage II – mild, 16-40 points = stage III – moderate, 40 + points = stage IV – severe. Figure 6 shows a reproduction of the classification of endometriosis from the "*Atlas of Laparoscopic and Hysterectomy Techniques*" by W.B.Saunders [96].

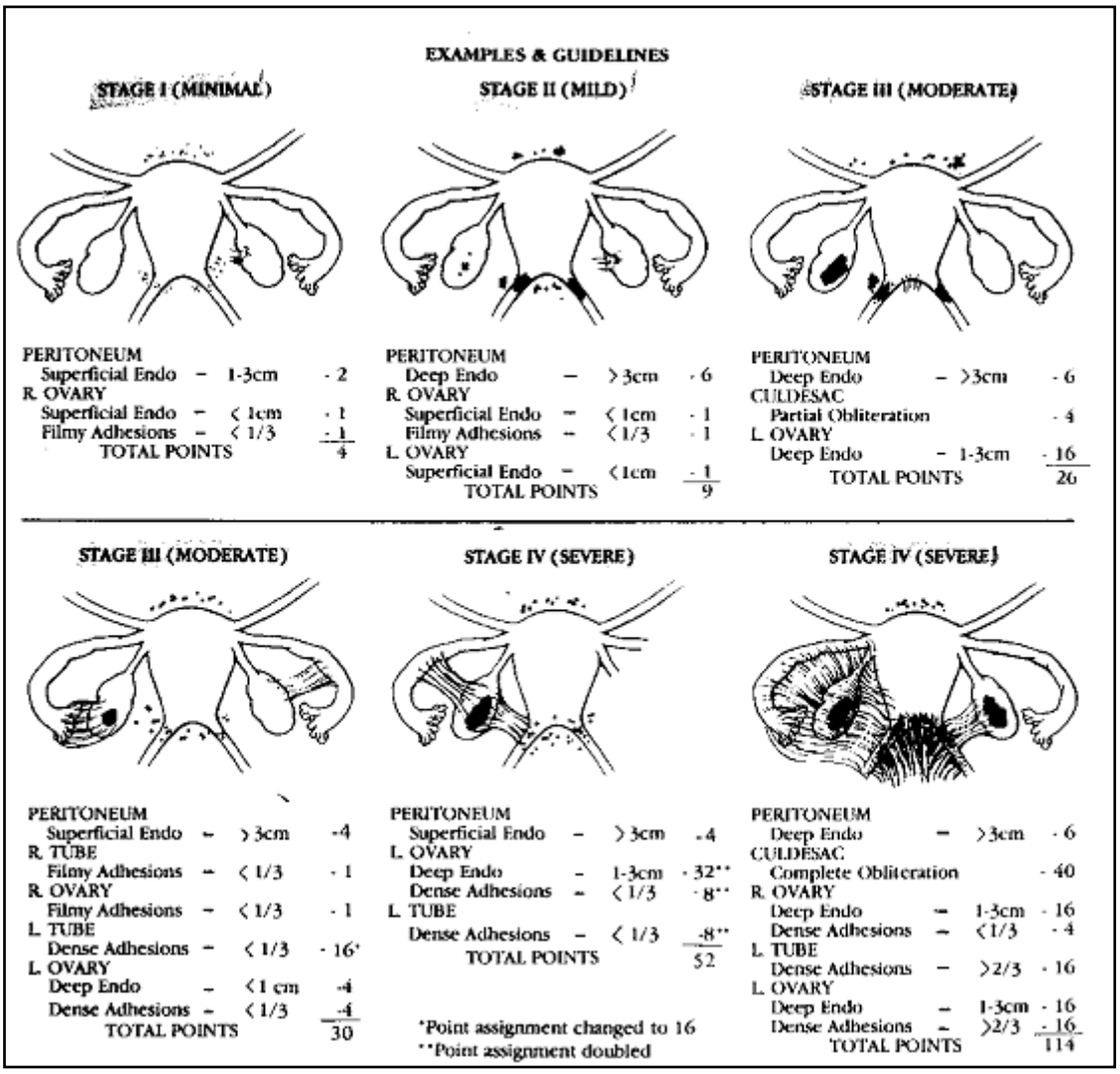


Figure 6. AFS staging criteria for endometriosis
Image reproduced from; *Atlas of Laparoscopic and Hysterectomy Techniques*, by W.B Saunders (1999) London Press

The American Society for Reproductive Medicine (ASRM) devised a grading system based on the appearance of endometriosis at laparoscopy. This is divided into four grades depending on the extent of endometriotic tissue present and are outlined in Table 3 below. The AFS criteria carries several advantages over the ASRM method, such as more detailed description of location and type of endometriotic lesion.

Table 3. Staging of endometriosis

Grade	**Description**
Grade 1	Possible Endometriosis – Peritoneal vesicles, red polyps, yellow polyps, hypervascularity, scar, adhesions.
Grade 2	Suggestive of endometriosis – Chocolate cysts with free flow of chocolate fluid. Chocolate cysts are endometriotic cysts filled with blood that has become brown with age.
Grade 3	Consistent with endometriosis – Dark scarred (puckered pigmented or mixed colour) lesions, red lesion on fibrous scarred background, chocolate cyst with mottled red and dark areas on white background.
Grade 4	Endometriosis – Dark, scarred (or puckered or pigmented) lesions at first surgery

Endometriosis can also be diagnosed histologically. At surgery a suspected area of endometriosis can be excised and examined microscopically for signs of endometriosis. The presence of endometrial glands and stroma infiltrating any tissue type is classified as endometriosis. Figure 7 Illustrates a classic example of histological endometriosis.

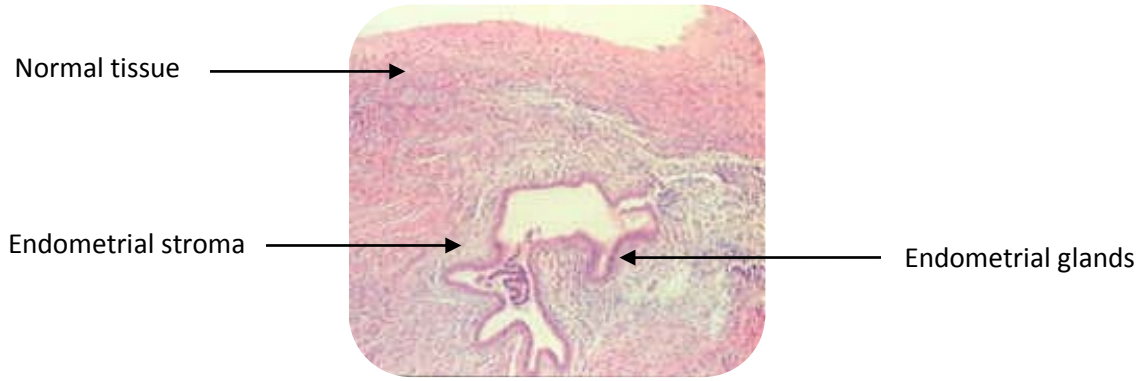


Figure 7. Microscopic view of endometriosis
Normal tissue i.e. epithelium of the uterus, is shown at the top of the image. An endometrial gland embedded in the uterine tissue is characteristic of endometriosis. Stromal tissue surrounds the outer border of the glandular tissue. Image courtesy of Current Medical Group, 2009

1.3 Current Medical Therapies for Endometriosis

Therapies for endometriosis are divided into two types of treatment, pharmacological therapies aim to inhibit growth of the endometriotic implants, whereas surgical therapies aim to remove or destroy the endometriotic implants [6].

1.3.1 Medical Therapies for Endometriosis

Endometriosis is an estrogen dependant disorder [97, 98], therefore current medical therapies are centred upon reducing circulating estrogen levels. This is usually achieved by down regulating ovarian production of steroid hormones (i.e. estradiol). Danazol, an isoxazol derivative of 17α-ethinyl testosterone (Figure 8) was the first drug to be approved for the treatment of endometriosis in the US [11]. Its anti-steroidogenic activity is known to relieve some of the symptoms associated with endometriosis [99], however substantial androgenic side effects, such as hair growth, mood changes and more seriously, liver damage and arterial thrombosis have been reported [100, 101].

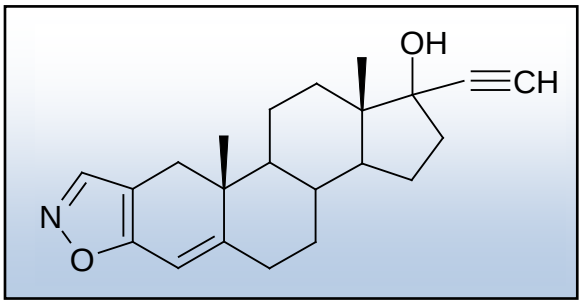


Figure 8. The structure of Danazol

1.3.1.1. Progestogens

The anti-estrogenic effects of progesterone have been mimicked in an effort to create medical therapies for endometriosis [6]. These compounds, known as progestogens, include Medroxyprogesterone and Norethindrone (Figure 9), which are derivatives of progesterone and 19-nortestosterone respectively. The drugs induce a pseudopregnancy state in which endogenous estrogen production is lowered. The mode of action of these drugs is thought to be due to their

suppression of the estrogen receptors, leading to endometrial decidualisation and eventual atrophy [102]. Additionally, these compounds have been shown to decrease the expression of matrix metalloproteinases, enzymes thought to be essential for the implantation and growth of ectopic endometrial cells [103]. Although these drugs are better tolerated than Danazol, their use still produces numerous side effects [104].

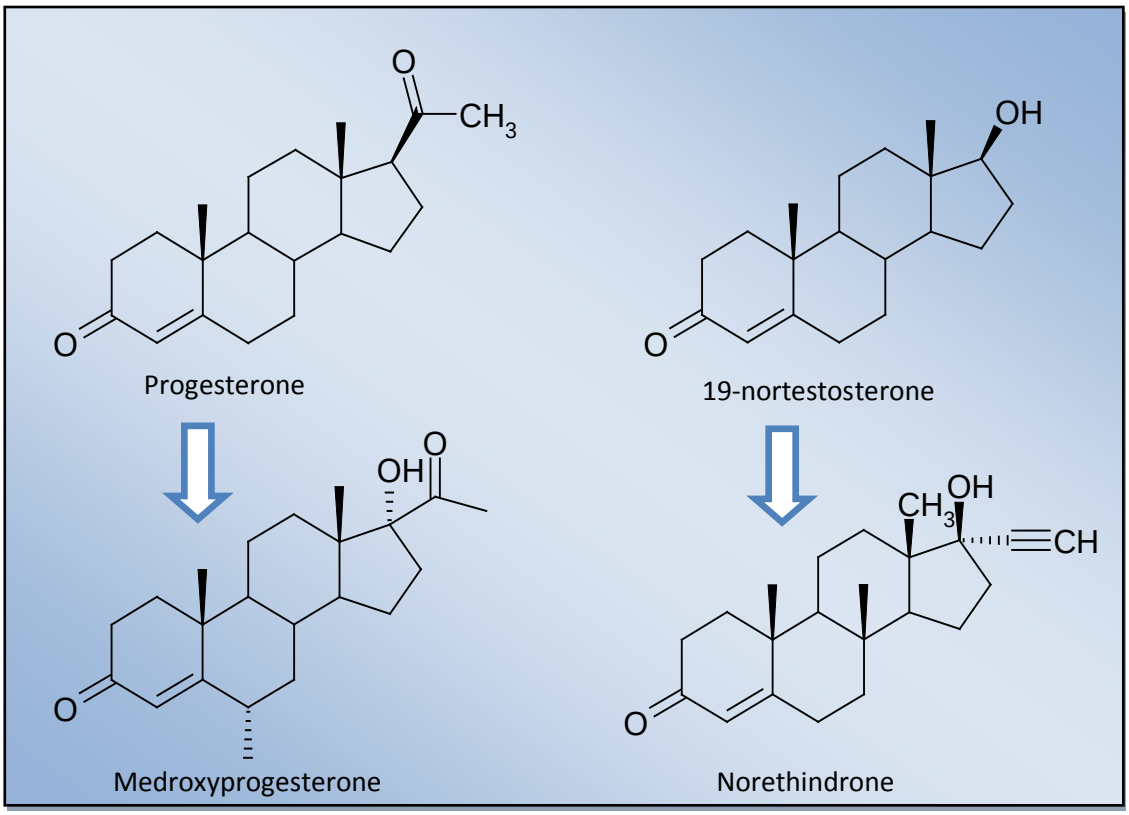


Figure 9. Progestogens Medroxyprogesterone and Norethindrone shown as derivatives of their conjugate steroid hormones Progesterone and 19-nortestosterone

1.3.1.2. GnRH Analogues

Gonadotrophin-releasing hormone (GnRH) agonists are analogs of the hypothalamic hormone GnRH. GnRH is responsible for the normal function of the ovaries by stimulating the release of follicle stimulating hormone (FSH) and luteneising hormone (LH) from the pituitary gland. GnRH agonists bind to pituitary receptors resulting in a shutdown of pituitary hormone secretion which in turn down regulates the ovarian production of estrogen, thus creating a hypoestrogenic state much like that which the body undergoes during menopause. Starving the endometriotic cells of estrogen in

combination with alterations of plasminogen activators and matrix metalloproteinases [105] is thought to result in endometriotic atrophy. However, endometriotic cells are known to express the aromatase enzyme [106], ensuring their survival independent of ovarian steroids.

GnRH agonist therapy is also associated with the side effects normally presented during menopause, such as hot flashes and loss of bone density, therefore in some cases small amounts of steroid hormone are administered in what is known as 'add back' therapy which appears to stem the severity of side effects without significantly affecting the relief of endometriosis associated pain [107].

1.3.1.3. Combined Oral Contraceptive

The combined oral contraceptive (COC) pill is commonly prescribed to women with endometriosis [108]. It consists of a combination of ethinyl estradiol and a progestin and induces a 'pseudopregnancy' state. Although data regarding the mode of action of COCs is sparse, initial investigation suggests they suppress proliferation and induce apoptosis of endometrial cells [109].

1.3.1.4. Aromatase Inhibitors

The reported ineffectiveness of hormonal therapy may be due to the resistant nature of endometriotic cells to progestogenic compounds. The under-expression of progesterone receptors [110] and the over-expression of estrogen receptors [111, 112] in endometriotic cells would render them less sensitive to current progestogenic therapies [113]. In some instances the aromatase enzyme may be activated in endometriotic cells [114], cells expressing this enzyme can produce their own estrogen supply, independent of ovarian steroids. Aromatase inhibiting compounds are currently being trialled for the treatment of endometriosis that is unresponsive to current therapy and have shown promising preliminary results [115].

Despite the success rates of various treatments for endometriosis, their use if often associated with numerous side effects and can only suppress ectopic endometrial cells [108]. Thus, discontinuation of treatment would likely lead to recurrence of disease symptoms. This assumption is supported by studies which identified that resurgence of endometriosis is common after treatment is halted [116]. A survey of 7,025 endometriosis patients by the *Endometriosis All Parliamentary Group* comprising women from 52 different countries found that hormonal therapies such as those mentioned above were only 37% effective at relieving symptoms [117]. However, the degree of effectiveness varied between different types of therapy. Interestingly, 39% of respondents found that hormonal therapy decreased their quality of life scores. It is also reported that 9% of women undergoing such therapy are completely unresponsive [118]. The side effects are unsurprising considering that most treatments have a whole body effect, not only affecting the ovaries, but also the brain and other cell types.

The reported over-expression of aromatase in endometriotic cells highlights how endometriotic cells appear so resilient and self sustaining. Aromatase expression is also found to be increased in certain cancerous cell types [119], in particular breast cancer [119, 120]. Aromatase inhibiting drugs such as letrozole and anastrozole are established therapies for breast cancer [121] and these drugs are currently undergoing clinical trial as a possible therapy for endometriosis, with some encouraging results [122-124]. However, the use of aromatase inhibitors is associated with the side effects of creating a dramatically hypoestrogenic environment, such as bone density loss [125].

1.3.1.5. Future Therapies

Due to the relatively poor efficacy of hormonal therapy for endometriosis, several other experimental therapies are currently undergoing clinical trial, such as Mifepristone (a selective progesterone receptor modulator) [126] and Dienogest (a progestin) [127]. Aromatase inhibitors have almost graduated from the pantheon of potential future therapies for endometriosis, since

they are now considered an acceptable therapy for endometriosis that does not respond to conventional treatments. However, there are other, novel medical therapies designed to exploit characteristics of endometriotic cells as potential drug targets. One such therapy is anti-angiogenic therapy [128]. Endometriotic tissue is thought to be derived from that of normal endometrium, which is a highly vascular tissue and one of the only such tissues in the adult human body that regularly undergoes the formation of new blood vessels. Support for this hypothesis comes from the observation that endometriotic implants are well supplied by vascular structures [129]. Animal models have also shown that endometriotic implants need an adequate angiogenic response in order to successfully survive [129, 130]. Several angiogenic factors, including the potent and well studied angiogenic factor vascular endothelial growth factor (VEGF), have been reported to be up regulated in endometriotic implants [131], endometrioma [132] and in the PF of women with endometriosis [133, 134]. Drugs that inhibit the formation of new blood vessels via a variety of mechanisms such as TNP-470, endostatin, VEGF-A blocking antibodies, methoxyestradiol and anginex, have all shown potential as anti-angiogenic therapies for endometriosis in animal models [135-139]. However, some drawbacks to this approach include the reduction in fertility observed in animals and patients taking anti-angiogenic therapy for cancer [140] and the possibility that not all the endometriotic tissue would be regressed by a single anti-angiogenic therapy owing to the heterogeneous nature of diseased tissue [135].

Recently Hassan et al [141] have suggested that gene therapy may be a novel approach to endometriosis treatment. Gene therapy involves delivering genetic material to diseased cells via a suitable vector in order to correct aberrant gene expression observed in disease cells. A study in 2002 by Dabrosin et al [142] used adenovirus vectors to deliver genes encoding the angiogenesis inhibitor, angiostatin, into mice engineered to develop endometriosis. This study reported 100% eradication of endometriotic implants in mice receiving the adenovirus compared to 15% reduction

observed in the control group. However, this study also reported a decrease in the indicators of fertility in adenovirus treated mice. Another approach suggested by Hassan et al [141] was to use adenovirus vectors to deliver dominant negative mutants of the estrogen receptor (DNER) to endometriotic cells. This would greatly reduce the sensitivity of endometriotic cells to estrogen and thus, atrophy their growth. Early results from this therapy have been encouraging, indicating increased cell death in endometriotic cells [143]. Such a treatment regime would confer advantages to the patient. For example, current hormonal therapy for endometriosis acts upon the whole body, often leading to many side effects. Gene therapy may also allow targeted therapeutics to specific cell types [144] and act at the cellular level greatly reducing unwanted side effects. Gene therapy however, is still in its infancy and suffers certain drawbacks such as rejection of the viral vectors by the host, short half life of the therapeutic DNA, and as only one gene can be corrected at a time, at present it is unsuitable for multifactoral diseases like endometriosis.

1.3.2 Surgical Therapy for Endometriosis

If medical therapy proves unsuccessful or the stage of endometriosis is considered too advanced then surgical intervention is often the only remaining option [6]. However, appropriate treatment varies significantly depending on the woman's age, parity and the nature of the symptoms. Surgery falls into two main categories, conservative surgery which can usually be performed via laparoscopy and radical surgery which normally involves the partial or entire removal of the affected organ.

1.3.2.1. Surgical Excision/Ablation of Endometriosis

The degree to which endometriotic implants cause pain is strongly related to the nature of the implant. It has been reported that deeply infiltrated endometriotic implants are most commonly associated with pain. Superficial implants on the other hand are often associated with minimal symptoms [145]. Excision of the endometriotic implant can be performed using a variety of techniques but all involve the cutting away of endometriotic tissue from healthy tissue. There are few well designed studies to assess the success of excisional therapy for the treatment of

endometriosis associated symptoms therefore it is hard to assess the benefits of this procedure over others. Additionally, complete excision of an endometriotic implant can be difficult. Koninckx et al [146] reported that despite an aggressive surgical approach, total removal of the implant could not be achieved more that 90% of the time [146], and even then complete removal of the implant was associated with serious complication in a quarter of cases.

Some surgeons forgo the use of excisional therapy in favour of ablative destruction of the endometriotic implant. This can be performed via a variety of techniques such as electrocautery or laser vaporisation. There are a number of reports on the use of ablation for managing the symptoms of endometriosis [147-149]. The overall conclusions that can be drawn from these studies indicate that surgical ablation has around a 60% success rate after 2 years (that is 60% of patients were pain free after 2 years). However, pain and individual's response to pain differs, affecting the conclusion that can be drawn from these studies.

1.3.2.2. Radical Surgery for Endometriosis

If management of endometriosis is not achievable by medical and minor surgical intervention, radical surgical options may have to be explored. Often this will involve the partial or complete removal of the affected organ. Most common procedures include hysterectomy (removal of the uterus) and oopherectomy (removal of the ovaries), although removal of the ovaries would require premenopausal patients to undergo hormone replacement therapy to lessen symptoms of estrogen deprivation. A seven year follow up study of 240 women who underwent surgery reported the following findings summarised in Table 4 on the effect of various surgical procedures for the relief of endometriosis associated pain based on the need for further surgery [150].

Table 4. Success of surgery related to endometriosis
Numbers relate to percentage of women who required further surgery

	Excision only	Hysterectomy only	Hysterectomy and oopherectomy
After 2 years	30.6%	4.3%	4%
After 5 years	46.7%	13.4%	8.3%
After 7 years	65.4%	23%	8.3%

Therefore, conclusions can be drawn from the result of this study. Firstly it would appear that surgical excision alone frequently requires further surgery suggesting a high rate of disease recurrence. Total hysterectomy with oopherectomy was the most successful surgery long term. This is most likely due to removing the source of potential refluxed endometrium (the uterus) and the source of mitogenic steroid hormones (the ovaries). Therefore, this also gives support to the retrograde menstruation theory, as it would appear removing the anatomical features nessacary for retrograde menstruation drastically reduces the recurrence of endometriosis. However, this study did not address issues such as what medical therapy, if any, the patients selected for this study were taking post-operatively, a factor which may influence the recurrence of the disease.

Chapter 2. A Novel Model for Predicting the Prevalence of Endometriosis

2.1. Background

The exact prevalence of endometriosis in any one country is difficult to predict with any degree of accuracy due to the invasive and subjective diagnostic procedures required to diagnose endometriosis. Although non-invasive methods for detecting endometriosis are currently under development, such as the CA125 antigen test [151], ultrasound scanning [152] and endometrial nerve density [153], definitive diagnosis of endometriosis can only be achieved visually via laparoscopy. However, diagnosis is a subjective matter depending on the skill of the surgeon and the quality of equipment used. Therefore, in poorer countries prevalence rates of endometriosis may be under represented due to lack of specialised doctors and poor quality surgical equipment. The 10% figure is often quoted as a universal constant for endometriosis prevalence. This may be erroneous however, given the heterogeneity of the world's populations the prevalence of endometriosis is likely to vary widely. Like many other diseases, prevalence of endometriosis will vary throughout the world but without a simple, objective diagnostic method the true prevalence of endometriosis within certain populations cannot be accurately measured. Endometriosis is a multifactoral disease, meaning it is highly unlikely a singular causative factor will ever be found. Like certain cancer types lifetime endometriosis risk is influenced by a number of factors. However, from the few epidemiological studies that have assessed endometriosis, certain factors can be used as indicators for the likelihood of endometriosis arising in populations where those factors are known. For example, the epidemiological data on endometriosis suggests that women with high parity, low meat consumption, high fruit and vegetable consumption, low alcohol consumption and live in a rural environment are less likely to develop endometriosis than those who have low parity, high meat consumption etc [70, 71]. Additionally, women with endometriosis have been shown to have an increased risk of developing ovarian cancer [154]. The reason for this is thought to be due to

endometriosis of the ovary representing a pre-malignant state in some cases [155, 156]. Whilst the prevalence of endometriosis in most of the world's countries may not be known, data on the above mentioned risk factors is well documented for most countries.

2.2. Aim

The aim of this study is to create a novel method for predicting the prevalence of endometriosis in different countries based on known risk factor data.

2.3. Methods

2.3.1. Collection of risk factor data

Data on the following risk factors for endometriosis was harvested for 165 countries:

1) Parity (average children per woman according to World Health Organisation 2004 [157]). The WHO collects metadata on parity from records of births averaged by the number of the female populace according to the latest population statistics for that country. WHO data has been used in a number of published peer reviewed articles [158, 159] and is thus deemed reliable.

2) Plant to animal matter consumption ratio (in calories per capita per day according to the Food and Agriculture Organisation metadata [160] 2001, calculated using the following equation, —

where Ap = mean consumption of total plant material for that country in calories per capita per day and $A\alpha$ = mean consumption of total animal material for that country in calories per capita per day). Plant matter is defined as the total consumption of the following food types classified by the FAO: fruit, vegetables, cereals, nuts and seeds. Animal matter is defined as the total consumption of the following food types as classified by the FAO: red meat (beef, lamb), white meat (chicken) and fish.

3) Alcohol consumption (in Kg per capita per year according to the Food and Agriculture Organisation [160] 2003). Alcohol is defined as the total consumption of the following alcohol types according to FAO classification: wine, beer and spirits. Consumption of plant matter, animal matter

and alcohol is calculated by the FAO by dividing consumption index by index of population. Metadata gathered by the FAO has been used in a number of peer reviewed articles [161, 162] and is therefore deemed reliable.

4) Percentage of population in urban environment (according to the World Health Organisation [157] 2005). Urban environment is defined by the WHO as any area where the population exceed or is equal to 1,500 people per square kilometre.

5) Incidence of ovarian cancer (incidence rates per 100,000 women according to Globocan 2002 [163]) were used to estimate the incidence of endometriosis for all countries where the data on risk factors were available. Globoan is a database of the CANCER*Mondial* website and is managed by the Cancer Information Section of the IARC (International Agency for Research on Cancer) in conjunction with the WHO. Cancer indices are gathered from international cancer registries. All statistics provided by Globocan are age-adjusted.

The sum of these risk factor data that are used for predicting the prevalence of endometriosis is presented in Appendix 1.

2.3.2. Prediction of Endometriosis

The risk factor data (Appendix 1) were used to predict the prevalence of endometriosis around the world. Countries that did not have data for all risk factors were eliminated from the analysis as they would represent incomplete data to obtain the prediction. Of the 165 countries, 44 were eliminated from the analysis. The reason these countries were eliminated was due to insufficient or missing data on the risk factors chosen for the prediction model. Difficulty arises when attempting to compare the data for each risk factor as each risk factor is given in units of measurement that are incompatible with one another, therefore a *rank scoring scale* had to be been established that gives the level of each risk factor for a certain country a comparative rank score out of five. The five ranks have been termed ‘very low’, ‘low’, ‘medium’, ‘high’ and ‘very high’ and correspond to the data

percentiles used in figures 10-15. However, firstly the risk factors were divided into *protective* and *suggestive* risk factors. Protective risk factors will decrease the incidence of endometriosis the higher their levels are i.e. fertility and plant to animal matter consumption ratio. Therefore, the rank scoring system for protective risk factors will be as demonstrated below

Rank	Very Low	Low	Medium	High	Very High
Score	5	4	3	2	1
Percentile	0-20	20-40	40-60	60-80	80-100

Conversely suggestive risk factors will increase the incidence of endometriosis the higher their levels are i.e. alcohol consumption, urban living and ovarian cancer incidence. Therefore, the rank scoring system for suggestive risk factors will be reversed, as demonstrated below.

Rank	Very Low	Low	Medium	High	Very High
Score	1	2	3	4	5
Percentile	0-20	20-40	40-60	60-80	80-100

Each country then has its score calculated based on the relevant rank it falls into for each risk factor. This data is supplied in Appendix 3. The maximum achievable score is 25, therefore the total score for each country was divided by 25 in order to obtain a relative endometriosis risk index (ERI). The ERI therefore ranges from 0 to 1. The closer a country's individual ERI is to 1 the higher predicted prevalence of endometriosis in that country. For example, an ERI of 0.95 would indicate that country has a high prevalence of endometriosis, conversely a score of 0.20 would indicate a low prevalence of endometriosis. The results of these data are presented in Figure 15.

2.3.3. Comparison of Age at Menarche and Human Development Index

There are also additional risk factors that can be taken into consideration but have less data available. For example, age at which menses begin (menarche) is considered to be one of the most important risk factors for endometriosis. Women who reach menarche earlier are more likely to develop endometriosis than those who have a later menarche [164-167]. The limited studies that have analysed data on age of menarche throughout various countries found that developed countries had a lower average age of menarche (e.g. Britain, 13.3 [168], Australia, 13 [169], Japan, 12.5 [170], USA, 12.8 [171] than developing countries (e.g. Somalia, 14.78 [172], Tanzania, 15.21 [173], Nigeria, 15 [169], Bangladesh, 15.8 [174], India, 14.31 [175]. The data above does not unequivocally prove that developing countries have a lower age of menarche though, it merely *infers* this conclusion. In order to prove the hypothesis that age of menarche (and therefore risk of endometriosis) is related to developmental status of a country firstly an indicator of a country's development status needs to be selected.

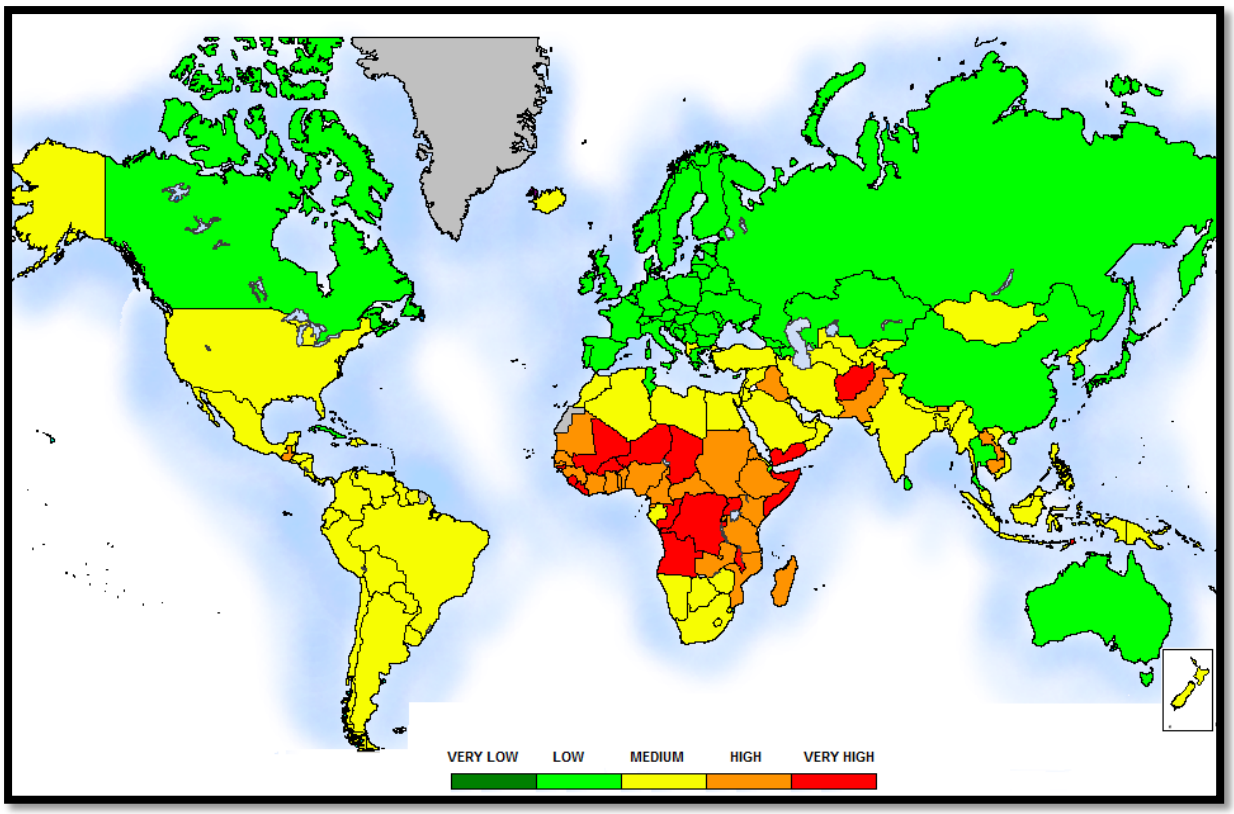
The Human Development Index (HDI) is a numerical value compiled by the United Nations Development Program (UNDP) given to a country represented by a value between 0 and 1. The HDI of a country is calculated by comparing normalised factors such as life expectancy, literacy and education indices and standard of living indices for a given country and reflects that country's development status. HDI data has been complied every 5 years since 1975. Age of menarche data has been compiled by Thomas et al and Morabia et al [169, 176]. Age at menarche data was date matched with HDI data for the closest year. Therefore, it is now possible to compare a country's developmental status with age of menarche. According to literature searches there has been no analysis to date that has investigated the relationship between age at menarche and the developmental state of a country. Since the data on age at menarche was compiled over several years it has be matched with HDI data for the closest year, a full listing of the data is presented in Appendix 2. Analysis of this data yielded some interesting results, firstly age at menarche was

plotted against HDI score (Figure 16), then Spearman's Rank correlation coefficient was calculated for the two data sets.

Using the HDI score it is also possible to compare the developmental state of a country to the predicted ERI score in order to test the hypothesis that developmental index is related to risk of endometriosis. In order to test this hypothesis ERI was plotted vs HDI values for 2006 for the 120 countries where both data was available. The results are plotted on Figure 17 and Spearman's Rank correlation calculated.

2.4. Results

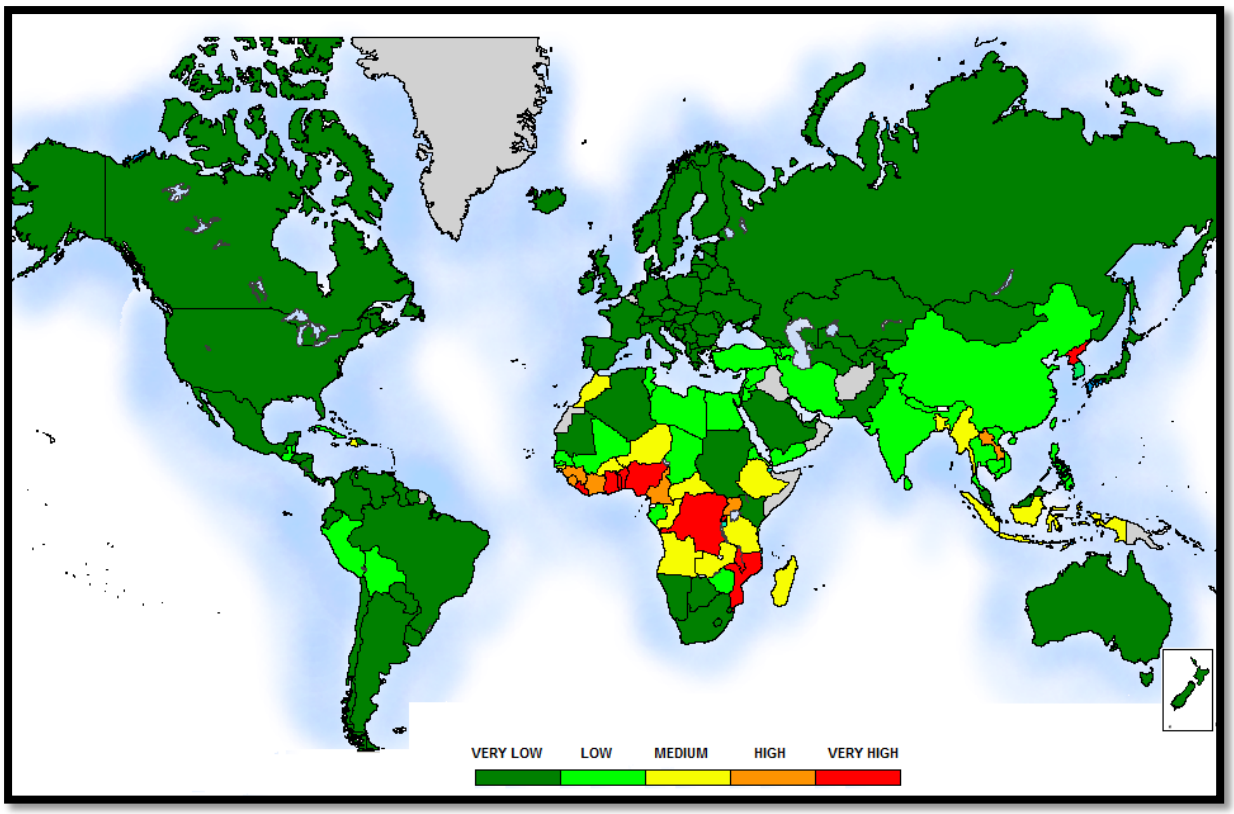
Analysis of the data shows some patterns in likely areas of endometriosis risk across the globe. In order to better visualise the tabulated data from Appendix 1 and Appendix 3 and judge patterns in risk, Figures 10-15 below represent a broad overview of the data obtained on maps of the world with colour coded levels of risk, a key is provided for each diagram explaining the risk levels. Countries where no data were available are represented as grey.



Risk Level	Description
Very low	Represents the 0-20th percentile of data i.e. those women who, on average have less than 1 child
Low	Represents the average 21-40th percentile of data i.e. those women who, on average have between 1 - 2.9 children
Medium	Represents the average 41-60th percentile of data i.e. those women who, on average have between 3 – 4.9 children
High	Represents the average 61-80th percentile of data i.e. those women who, on average have between 5 – 6.9 children
Very High	Represents the average 81-100th percentile of data i.e. those women who, on average have over 7 children

Figure 10. Risk Factor - Fertility

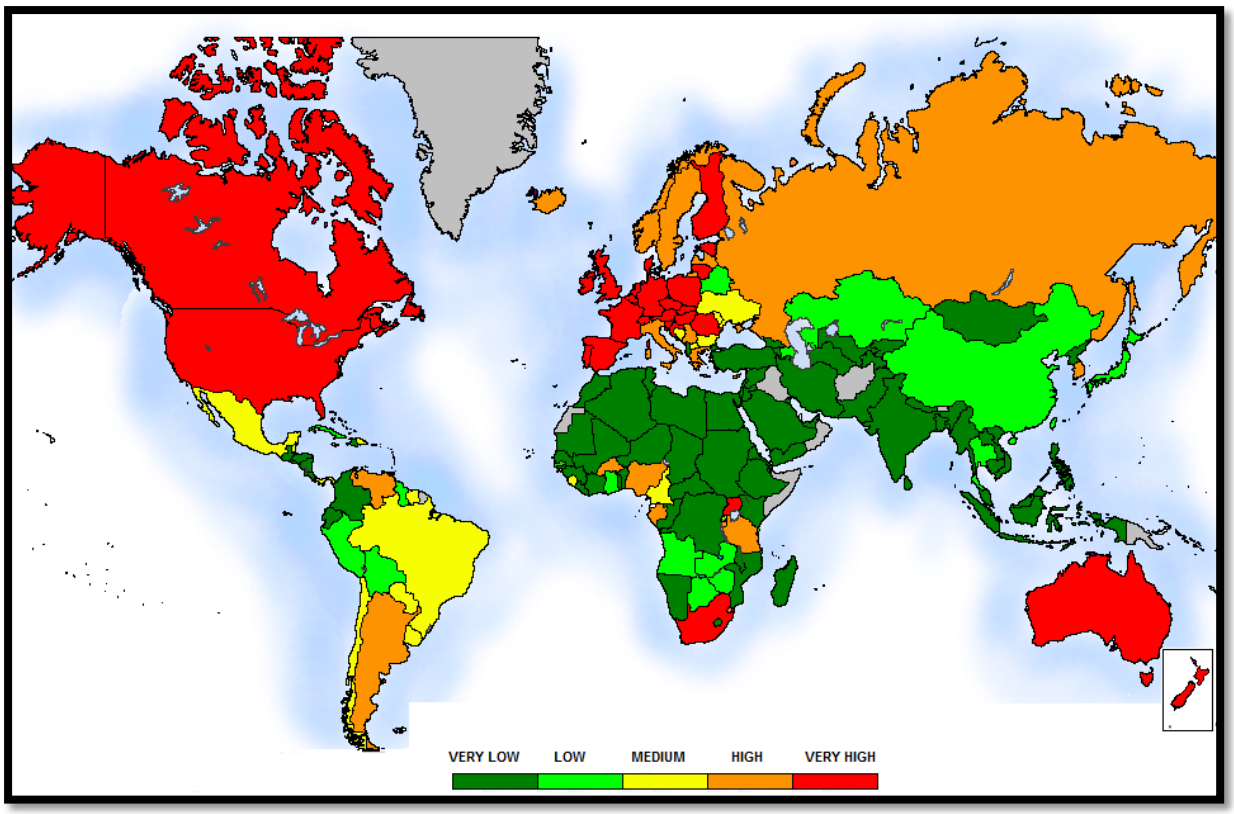
Worldwide the mean fertility rate is 2.9 ± 1.6 children per woman. Figure 10 shows fertility rates are highest in Central Africa, the Middle East and Southern Asia. Highest fertility rates were recorded in Niger (7.8 children per woman) and Uganda (7.1). Fertility rates are lowest in Europe, China and Canada. Lowest fertility rates were recorded in Ukraine (1.1). Figure 21 shows the relationship between predicted Endometriosis Risk Indices and fertility data.



Risk Level	Description
Very low	Represents the 0-20th percentile of data i.e. those populations who have a plant to animal consumption ratio of 0-2.9
Low	Represents the average 21-40th percentile of data i.e. those populations who have a plant to animal consumption ratio of 3-5.9
Medium	Represents the average 41-60th percentile of data i.e. those populations who have a plant to animal consumption ratio of 6-9.9
High	Represents the average 61-80th percentile of data i.e. those populations who have a plant to animal consumption ratio of 10-12.9
Very High	Represents the average 81-100th percentile of data i.e. those populations who have a plant to animal consumption ratio of 13-15+

Figure 11. Risk Factor – Plant to Animal Matter Consumption Ratio

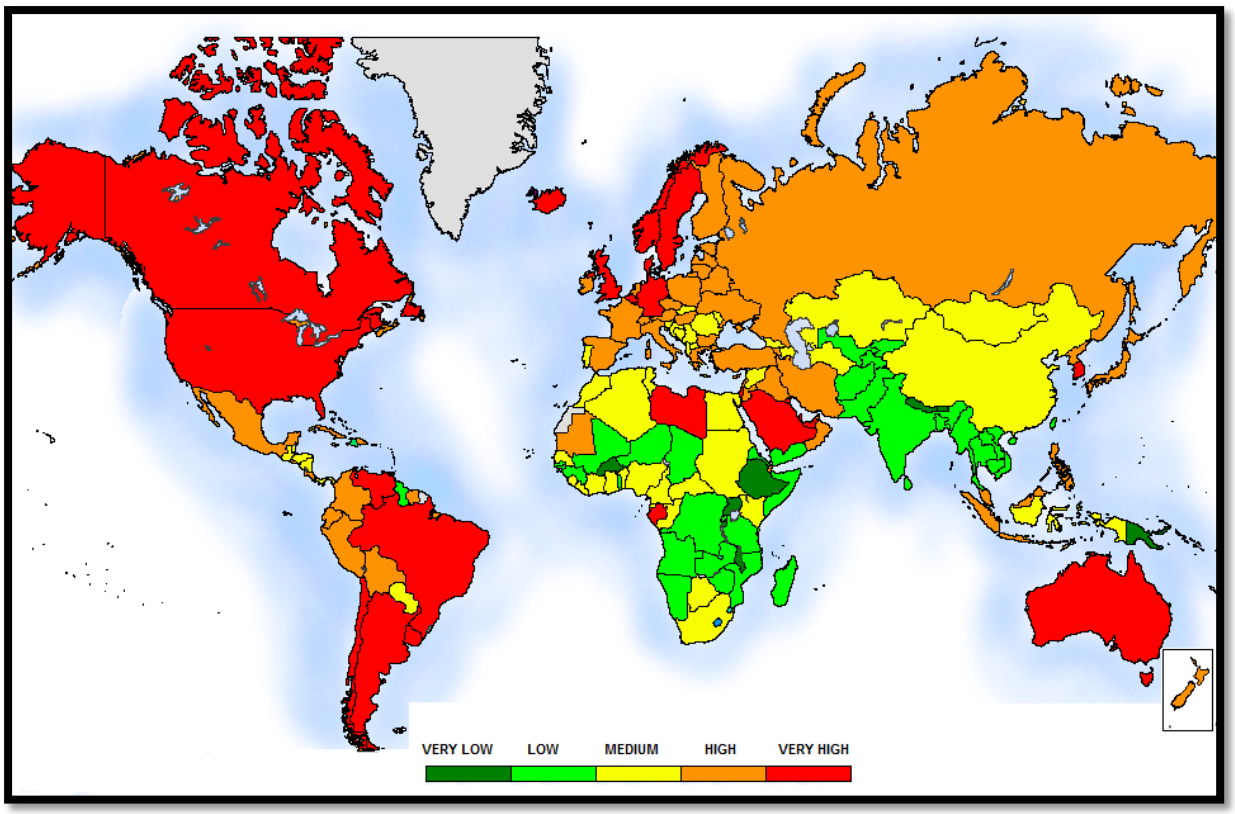
Worldwide the mean plant to animal matter consumption ratio was 8.3 ± 8.5. Figure 11 indicates plant matter constitutes a higher proportion of the diet of people in central Africa and North Korea. Highest levels of plant matter consumption were recorded in Democratic republic of Congo (ratio 45.57) and Mozambique (42.84). Meat constitutes a greater proportion of the diet in Northern Europe, Australia, North and South America, the Middle East and South Africa. Highest meat consumption was recorded in Iceland (ratio 1.45) and Denmark (1.53). Figure 22 shows the relationship between predicted ERI scores and dietary plant to animal matter consumption ratios.



Risk Level	Description
Very low	Represents the 0-20th percentile of data i.e. those populations who have an average alcohol consumption of between 0 – 20.9 Kg/capita/year
Low	Represents the average 21-40th percentile of data i.e. those populations who have an average alcohol consumption of between 21 – 40.9 Kg/capita/year
Medium	Represents the average 41-60th percentile of data i.e. those populations who have an average alcohol consumption of between 41 – 60.9 Kg/capita/year
High	Represents the average 61-80th percentile of data i.e. those populations who have an average alcohol consumption of between 61 – 80.9 Kg/capita/year
Very High	Represents the average 81-100th percentile of data i.e. those populations who have an average alcohol consumption of between 81 – 100+ Kg/capita/year

Figure 12. Risk Factor – Alcohol Consumption

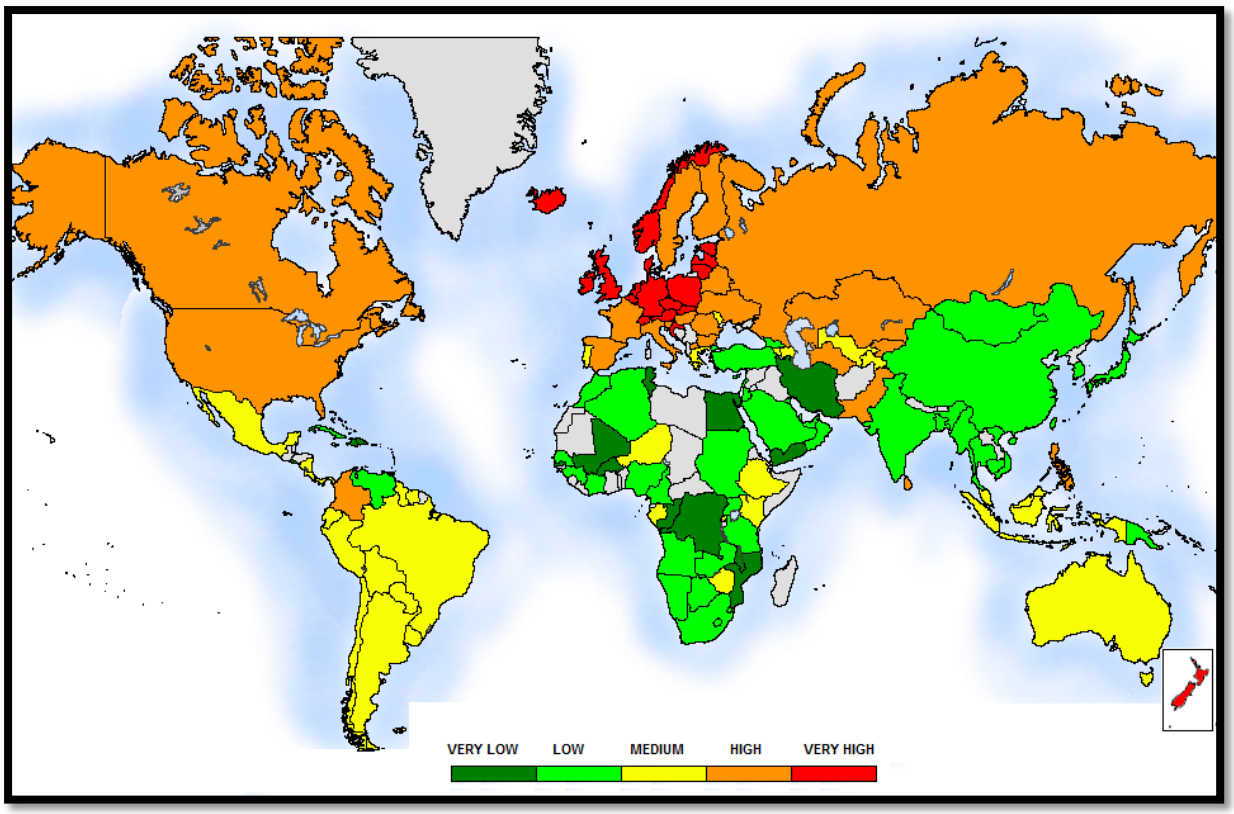
Worldwide the mean alcohol consumption is 46.7 Kg/capita/year ± 44.7. Figure 12 shows alcohol consumption is highest in Europe, North America, Australia and South Africa. Highest levels of alcohol consumption were recorded in Ireland (211 Kg/capita/year). Intermediate areas of alcohol consumption include South and Central America. The lowest level of alcohol consumption was recorded in Bangladesh, Indonesia, Iran, Kuwait, Myanmar, Niger, Pakistan, Saudi Arabia, Sudan and Yemen (0 Kg/capita/year). Figure 23 shows the relationship between predicted ERI scores and alcohol consumption data.



Risk Level	Description
Very low	Represents the 0-20th percentile of data i.e. those populations where on average 0-20% of the populace live in an urban environment
Low	Represents the average 21-40th percentile of data i.e. those populations where on average 21-40% of the populace live in an urban environment
Medium	Represents the average 41-60th percentile of data i.e. those populations where on average 41-60% of the populace live in an urban environment
High	Represents the average 61-80th percentile of data i.e. those populations where on average 61-80% of the populace live in an urban environment
Very High	Represents the average 81-100th percentile of data i.e. those populations where on average 81-100% of the populace live in an urban environment

Figure 13. Risk Factor – Urban Living

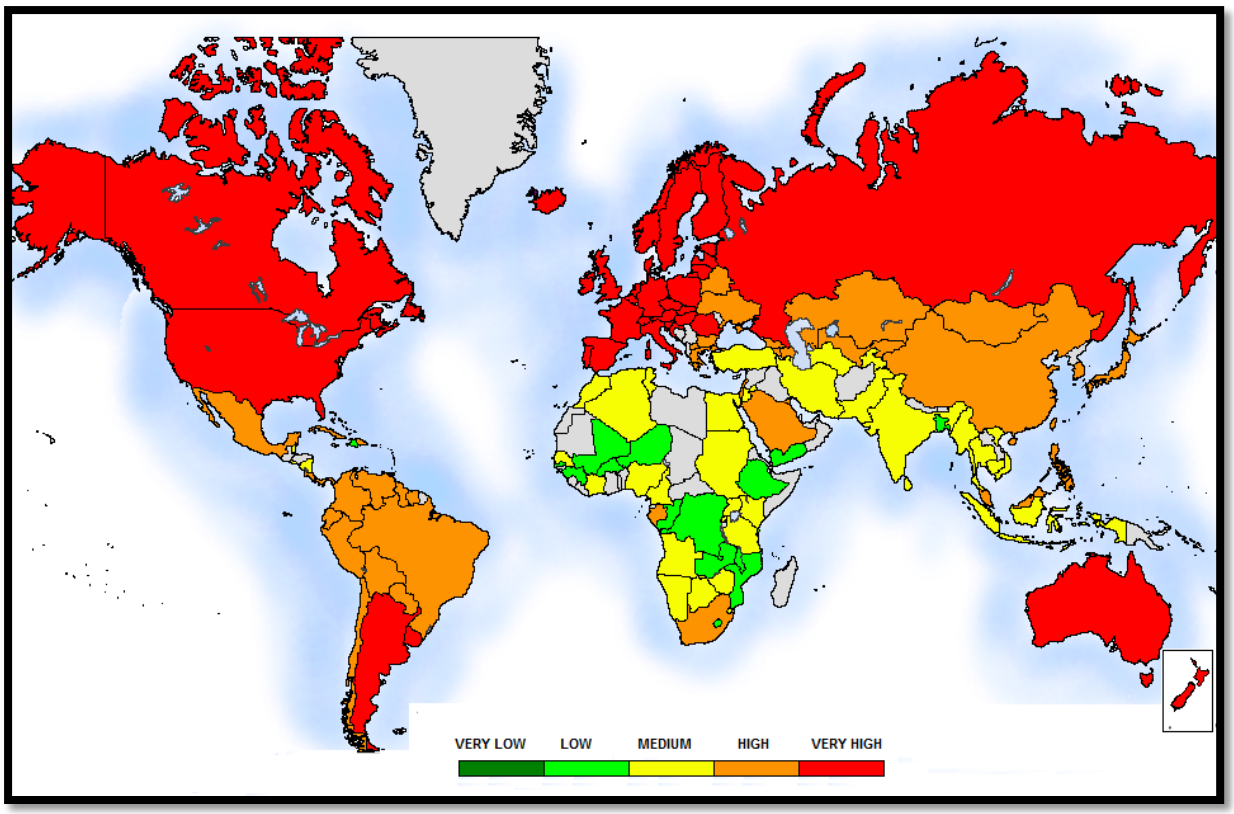
Worldwide the mean percentage of people who live in urban environments is 57.3% ± 21.7. Figure 13 shows the majority of the populace of North America, South America, Northern Europe and Australia live in urban environments. The highest % of urban population was found in Belgium (97.3%) and Kuwait (96.4%). Around half the population of China and Central Africa live in urban areas. The lowest rates of urban living are found in sub-equatorial Africa and Southern Asia. The lowest rates of urban population were found in Uganda (12.4%) and Ethiopia (16.2%). Figure 24 shows the relationship between predicted ERI scores and percentage of population living in urban environments.



Risk Level	Description
Very low	Represents the 0-20th percentile of data i.e. those populations where the average ovarian cancer incidence is between 0 – 2.9 cases per 100,000 women
Low	Represents the average 21-40th percentile of data i.e. those populations where the average ovarian cancer incidence is between 3 – 5.9 cases per 100,000 women
Medium	Represents the average 41-60th percentile of data i.e. those populations where the average ovarian cancer incidence is between 6 – 8.9 cases per 100,000 women
High	Represents the average 61-80th percentile of data i.e. those populations where the average ovarian cancer incidence is between 9 – 11.9 cases per 100,000 women
Very High	Represents the average 81-100th percentile of data i.e. those populations where the average ovarian cancer incidence is between 12 – 15+ cases per 100,000 women

Figure 14. Risk Factor – Ovarian Cancer

Worldwide the mean incidence of ovarian cancer is 7.4 cases per 100,000 ±3.7 (2002). Figure 14 shows ovarian cancer incidence rates are high in North America, Southern and Eastern and Northern Europe. Highest incidences were reported in Iceland (17.0 cases per 100,000) and Lithuania (16.6 cases per 100,000). Areas where ovarian cancer incidences are around the mean include South America and Australia. Lowest Ovarian cancer rates are found in Central and sub-equatorial Africa as well as South East Asia. The lowest incidence was reported in Egypt (1.0 cases per 100,000). Figure 25 shows the relationship between predicted ERI scores and rates of ovarian cancer.



Risk Level	Description
Very low	Represents those countries with an Endometriosis Risk Index between 0.00 – 0.19
Low	Represents those countries with an Endometriosis Risk Index between 0.2 – 0.39
Medium	Represents those countries with an Endometriosis Risk Index between 0.4 – 0.59
High	Represents those countries with an Endometriosis Risk Index between 0.6 – 0.79
Very High	Represents those countries with an Endometriosis Risk Index between 0.8 - 1

Figure 15. Predictive Endometriosis Prevalence

Figure 15 show the sum of predicted Endometriosis Risk Indices worldwide. It indicates endometriosis prevalence is highest in North America, Europe and Australia, being highest in Belgium, Germany, United Kingdom and New Zealand (ERI 0.96. Intermediate areas of predicted endometriosis risk include North Africa, South Asia and Central America. Lowest areas of predicted endometriosis risk occur around Central Africa, being lowest in Malawi (ERI 0.24). The mean ERI worldwide is 0.64 ± 0.20.

Figure 16 displays the correlation between HDI and Age at menarche for countries throughout the world with linear regression.

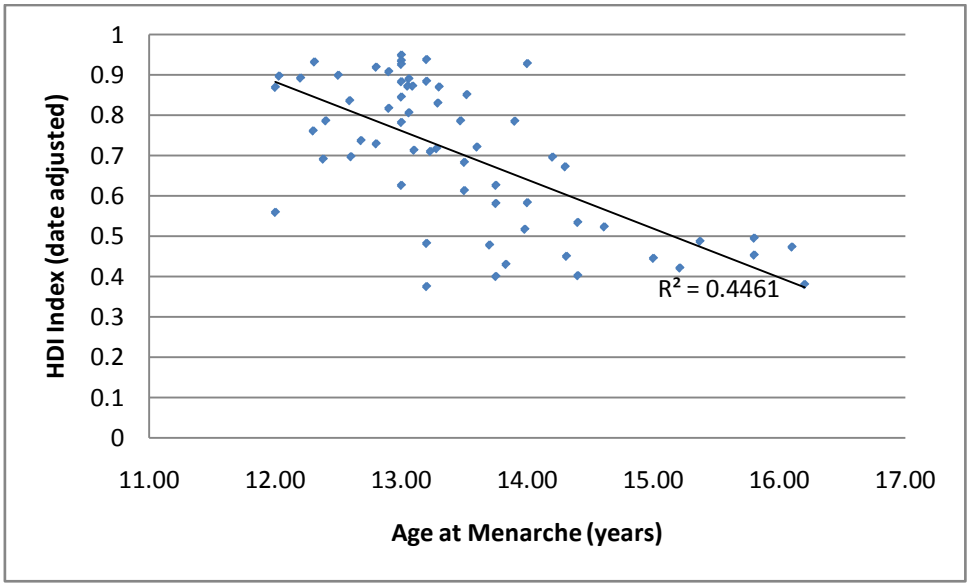


Figure 16. Age at menarche vs Human Development Index

Spearman Rank correlation = -0.6343 which is indicative of a weak negative relationship, however the critical P value for this result is <0.001 indicating that although this was a weak correlation it was highly statistically significant.

Figure 17 displays the correlation between HDI and the predicted Endometriosis Risk Index (ERI) with linear regression.

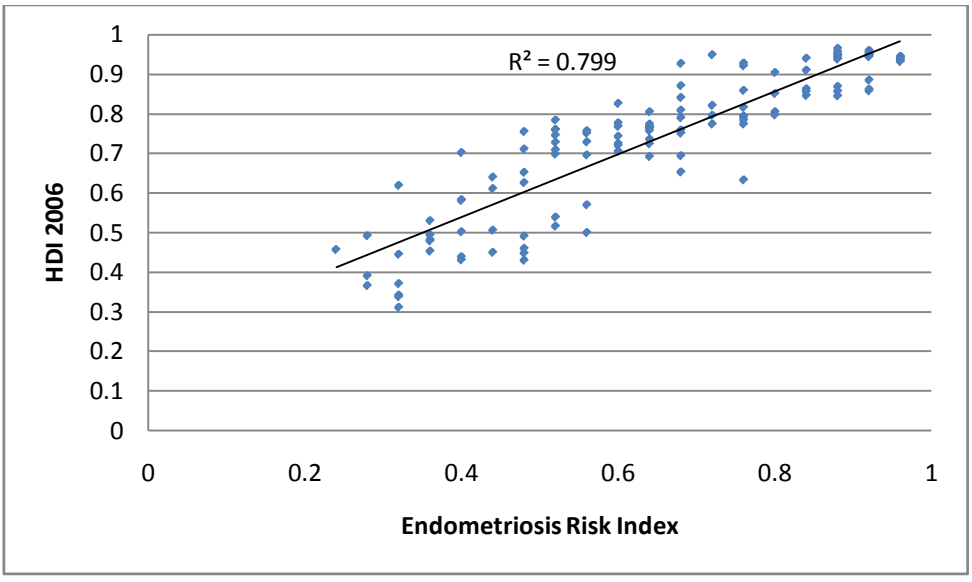


Figure 17. Human Development Index vs Endometriosis Risk Index

Although the R^2 value of 0.799 indicates a strong correlation, Spearman Rank correlation = 0.9133 which is indicative of a very strong, statistically significant association. Therefore, age at menarche is associated strongly with risk of endometriosis and age at menarche is inversely related to HDI.

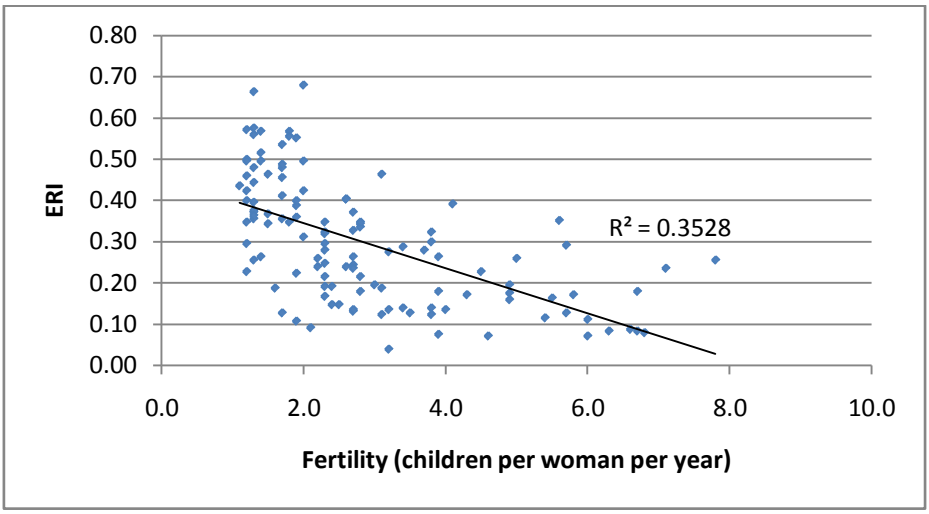


Figure 18. Endometriosis Risk Index vs Fertility

Linear regression gives an R^2 value of 0.3528 indicating a weak correlation between the predicted ERI values and Fertility rates. Spearman Rank correlation = -0.797 indicating a moderate negative correlation with critical P values <0.001 indicating it is statistically significant.

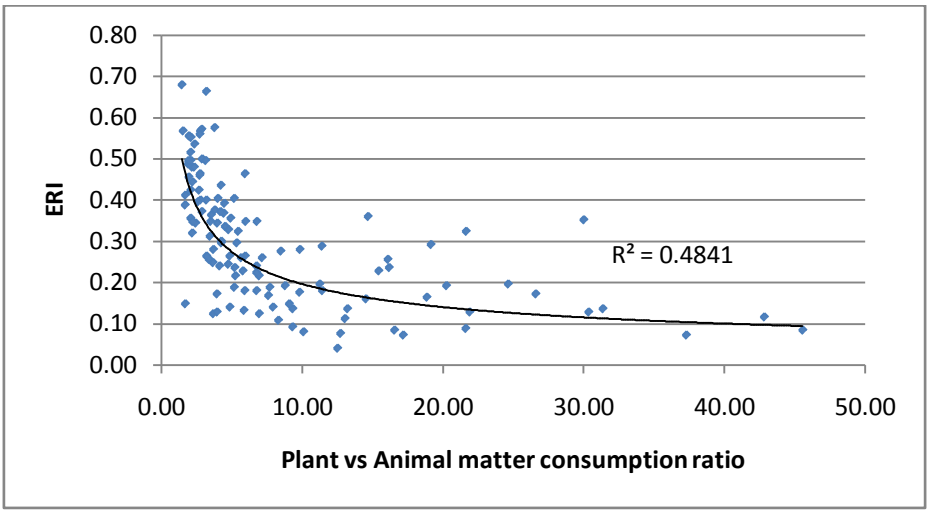


Figure 19. Endometriosis Risk Index vs Plant:Animal matter ratio

Logarithmic regression gives an R^2 value of 0.4841 indicating a weak correlation between the predicted ERI values and Plant: Animal matter consumption ratios. Spearman Rank correlation =

-0.9121 indicating a strong negative correlation with critical P values <0.001 indicating it is statistically significant.

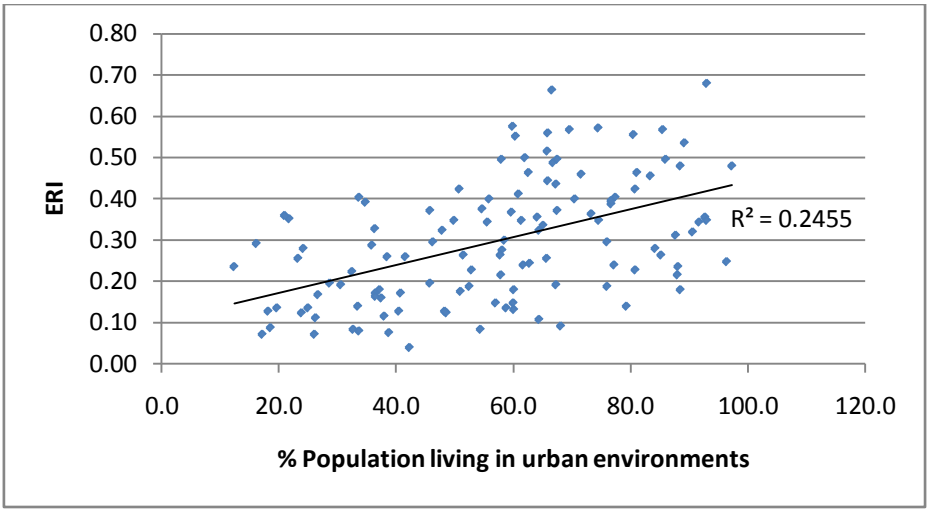


Figure 20. Endometriosis Risk Index vs Urban living

Linear regression gives an R^2 value of 0.2455 indicating a very weak correlation between the predicted ERI values and percentage population living in urban environments. Spearman Rank correlation = 0.7747 indicating a moderate positive correlation with critical P values <0.001 indicating it is statistically significant.

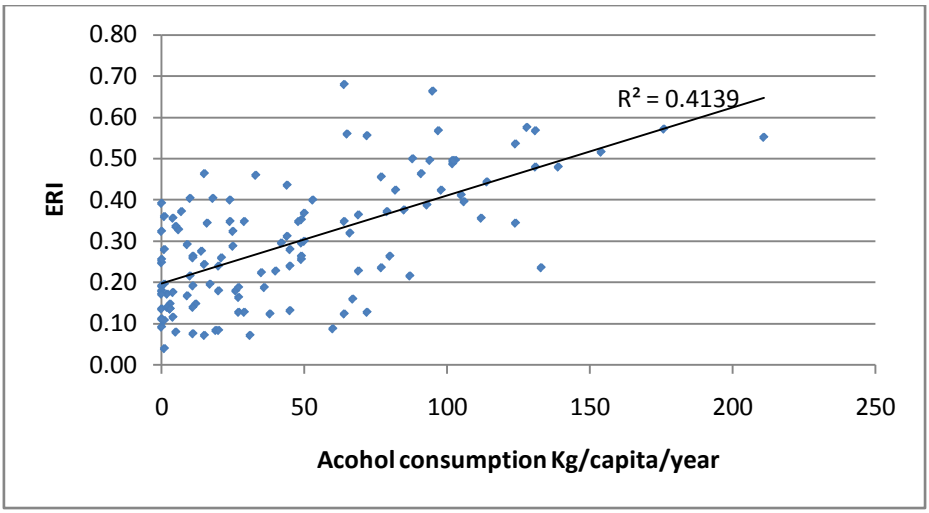


Figure 21. Endometriosis Risk Index vs Alcohol consumption

Linear regression gives an R^2 value of 0.4139 indicating a weak correlation between the predicted ERI values and alcohol consumption. Spearman Rank correlation = 0.7481 indicating a moderate positive correlation with critical P values <0.001 indicating it is statistically significant.

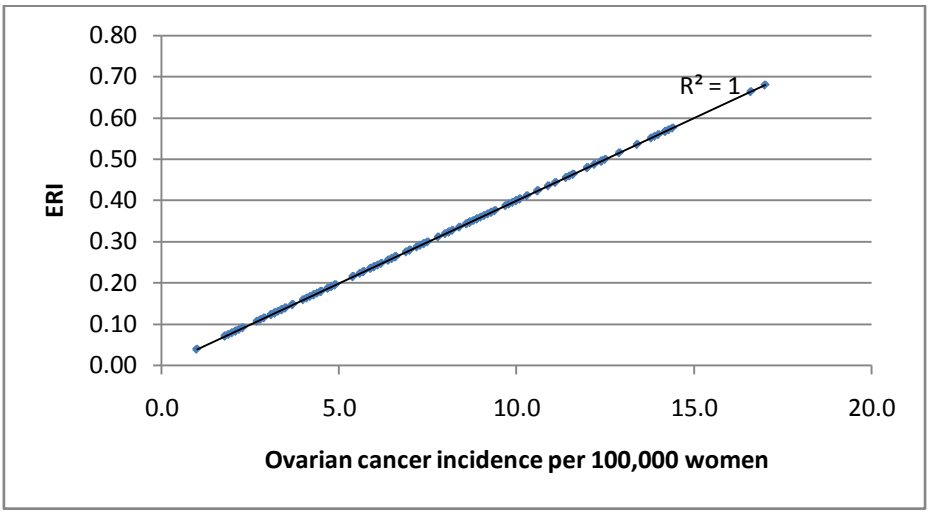


Figure 22. Endometriosis Risk Index vs Ovarian cancer incidence

Linear regression gives an R^2 value of 1.00 indicating a perfect correlation between the predicted ERI values and ovarian cancer incidence. Spearman Rank correlation = 0.8208 indicating a strong positive correlation with critical P values <0.001 indicating it is statistically significant.

2.5. Discussion

As the true prevalence of endometriosis throughout countries of the world is unknown, a prediction model such as the one outlined in this chapter is our best method of estimating endometriosis prevalence. This is the first study of its kind to attempt to provide a satisfactory endometriosis prediction model.

Choosing the correct risk factors for this study is essential, yet this study has been limited to risk factors where data is well documented. Other risk factors have been suggested for increasing the

incidence of endometriosis, such as light skin pigmentation [177, 178], menstrual characteristics [54, 179] and specific pollutant exposure [75, 82] however, accurate data on such risk factors is either scant or non-existant. Each risk factor has been selected based on evidence linking each one to either a decrease or increase in risk of developing endometriosis.

Figure 18 shows that there is a weak negative relationship between the predicted ERI score and number of children. Parity has long been recognised as a protective factor [166, 180]. The reason for this is thought to be due to the suppression of ovarian estrogen production during pregnancy, effectively starving any endometriotic lesions of their main trophic factor. Although pregnancy is often touted as a cure for endometriosis this is a fallacy, as endometriosis can recur postnatally. In point of fact a retrospective study of 345 women with endometriosis found that recurrence of disease symptoms was significantly *increased* after the birth of the first child [181].

Figure 19 shows that there is a slightly more significant relationship between predicted ERI scores and plant: animal matter consumption ratio. Greater consumption of plant matter appears to be associated weakly with a lowered risk of endometriosis. Epidemiological data has suggested that diet plays a role in endometriosis susceptibility, specifically consumption of fruit and vegetables reportedly decreases risk whereas meat consumption, particularly red meat, increases risk [70, 71]. High plant matter consumption may be an indicator of a generally healthy lifestyle, thus lowering overall disease rates. However, studies indicate a diet high in fruit and vegetables is protective against endometrial cancer [182], fibroids [183] and epithelial ovarian tumours [184], all of which are thought to be associated with estrogen dependency and endometriosis [185-187]. The protective effect of plant matter may be due to higher concentrations of vitamin C, carotenoids, folic acid, micronutrients and phytoestrogens present in such foodstuffs, which may decrease cellular

proliferation [188, 189]. Animal matter, in particular animal fats, are thought to increase the production of prostaglandins and increase the production of circulating estrogens [190, 191]. Additionally, the heterocyclic amines, residual hormones and growth enhancers found in red meat are thought to act as estrogenic compounds [192, 193] which may increase the proliferation of endometriotic implants.

Figure 20 shows that there is only a very weak association between predicted ERI scores and urban living. According to these results urban living is not associated with any significant increase in endometriosis risk. Living in urban environments has been suggested as a risk factor for endometriosis [164, 180], as it is thought that increased concentrations of endocrine disrupting chemicals (EDCs) present in pollution associated with dense urban areas may contribute to the development of endometriosis [73]. EDCs are chemicals that can interfere with normal hormonal signalling systems, either by blocking or modulating the synthesis, transport, binding and metabolism of endogenous hormones. Dioxin and polychlorinated biphenyls (PCBs) are the EDCs mostly cited as involved in endometriosis risk due to estrogenic activity [75, 82], although the association between such EDCs and endometriosis is tenuous (see section 1.1.5). Similarly, the EDC properties of phthalate esters have been associated with endometriosis in some studies [194, 195], but not others [196]. Phthalate esters are ubiquitous environmental pollutants known to be found in high concentrations in urban environments [197, 198]. The source of phthalate exposure is predominately plastic food containers but other sources include; plastic flooring (vinyl), pharmaceutical coatings and airborne dust. Further discussion on the transgenerational effects of EDCs is presented in section 3.1.5. Associations have also been made between endometriosis risk and lead pollution [199], which is known to be high (between 180-200ng/m^3) in urbanised areas, mainly due to vehicle emissions [200-202].

Figure 21 shows that there is a weak association between predicted ERI scores and alcohol consumption. Alcohol consumption has been cited as a risk factor for endometriosis [72, 164, 165, 203], although not all studies are concordant [204]. Those that show a correlation between endometriosis and alcohol only note alcohol consumption of the sufferer, which may not be a causative factor. Rather endometriosis may be a causative factor for increased alcohol consumption due to the analgesic effects of alcohol. Some of the above studies reported intrauterine exposure to alcohol did not result in any subsequent increase in endometriosis risk. However, this study was based on retrospective data recollection by the sufferer's mothers that may hinder accurate data collection due to each sufferer being between the ages of 18-40. Additionally, inheritance of endometriosis from the mother was not controlled for. Alcohol is thought to increase the risk of endometriosis by increasing circulating estrogen levels [205] and increasing the expression of estrogen receptors [206]. Red wine has also been reported to increase the expression of aromatase in adipose tissue [207], which may further contribute to increased estrogen production. Due to the extensive, reliable data collected on alcohol consumption by the FAO it was included as a predictive risk factor for endometriosis for this study.

Figure 22 shows a perfect correlation between predicted ERI scores and ovarian cancer incidence. This result is unusual because of its perfect correlation, one would expect that being as the ERI scores are derived from five separate factors, there would be no one factor with perfect correlation. Therefore, this result should be treated with caution and is most likely an artefact. Although it is considered a rare event, endometriosis of the ovary (endometrioma) has long been recognised as possessing the ability to transform into a malignant state, in particular epithelial and clear cell carcinoma [208]. Cancer is recognised as a stepwise accumulation of genetic lesions that eventually result in malignancy [209]. Endometrioma may be considered one of the intermediate steps between normal tissue and malignancy due to similar genetic risk factors associated with

endometriosis and cancer [210]. Therefore, although some variation is likely to exist, it is reasonable to conclude that countries which have a higher incidence of ovarian cancer would also have a higher incidence of endometriosis. Given the wealth of data on global ovarian cancer incidence collected by the WHO it is reasonable to have included it as a risk factor for this study. The relationship between endometriosis and cancer is discussed further in section 3.5.

Countries with the highest ERI score (0.96) were Belgium, Denmark, Germany, New Zealand and United Kingdom. Countries that fell along the mean ERI value (0.64) were Armenia, Azerbaijan, Bolivia, Ecuador, Lebanon, Malaysia, Paraguay, Peru, Philippines and Turkmenistan. The country with the lowest ERI score (0.24) was Malawi preceded closely by Yemen, Mozambique and the Democratic Republic of the Congo with an ERI score of 0.28. Analysis of these data reveals obvious trends in patterns of endometriosis risk. For instance, countries across the equator, in particular central Africa appear likely to have low prevalence's of endometriosis (i.e. likely much lower than the standard 10% prevalence rate) due to consistently high protective factors and low suggestive factors. The converse can be said for developed countries such as those in North America and Europe (in particular Northern Europe), which appear likely to have high prevalence's of endometriosis (i.e. equal to or higher than the standard 10% rate) based on the risk factors analysed. Southern America, China, Indonesia and Southern Asia appear to be intermediate areas regarding endometriosis risk (i.e. Higher than Africa but lower that Northern Europe).

The data on age at menarche shows a clear correlation between age at menarche and HDI. Due to early menarche being a major indicator of endometriosis risk it can thus be concluded that risk of endometriosis is related to HDI. This notion is supportive of the accuracy of the prediction model as HDI is also correlated with the theoretical ERI score (Figure 17). This is the first study of its kind to demonstrate a link between HDI and age at menarche.

The evidence presented in this chapter provides substantiation for the conclusions proposed above which indicate that women living in developed countries have a higher risk of endometriosis than those in developing nations. This is supported by the existing literature, which suggests that women of native African nationality are at the lowest risk of endometriosis whilst Caucasian European (particularly Northern Europeans) women at the highest risk. Hispanics and Asians were documented as having an intermediate level of risk [2-4], which provides further evidence for the accuracy of this model. Yet validation of its accuracy can only come as further real world data is gathered. It would be of significant interest to document any countries whose ERI score did not match those of real world data. Analysis of the demographic and lifestyle of a population of any such country may provide additional insight into factors underlying the development of endometriosis.

Testing this prediction model would require the extrapolation of predicted prevalence data for a certain country and comparing this to actual data. Deriving an estimate of endometriosis prevalence for a certain country can be achieved by using the ERI score as a proportional representation of the prevalence of endometriosis in any given population of women of reproductive age. For example, it is widely reported that countries such as the United Kingdom and New Zealand have an endometriosis prevalence of 10% of women of reproductive age. Both these countries have an ERI score of 0.96, therefore it is reasonable to conclude that countries with of ERI of 0.96 will have an endometriosis prevalence of 10%. If it is supposed that endometriosis prevalence decreases proportionally with ERI then we have a method of producing testable endometriosis prevalence's (represented by a percentage of population of females of reproductive age) from this prediction model. Taking this further, if the estimated prevalence's of endometriosis are known, then it would be possible to estimate the number of endometriosis sufferers in any country for which the ERI is known. Table 5 below illustrates some examples of predicted endometriosis prevalence's for selected countries.

Table 5. Predicted Endometriosis Prevalence for Selected Countries

Country	ERI Score	Predicted Endometriosis Prevalence (%)	Female Population of Reproductive Age (15-60) [211]	Predicted Endometriosis Prevalence (individuals)
United Kingdom	0.96	10.0%	20,185,040	2,018,504
United States	0.92	9.6%	102,161,823	9,790,508
Brazil	0.76	7.9%	66,157,812	5,237,493
Japan	0.72	7.5%	40,894,057	3,067,054
China	0.60	6.3%	465,020,030	29,063,752
Pakistan	0.52	5.4%	48,921,023	2,649,889
India	0.44	4.6%	352,868,003	16,173,117
Bangladesh	0.36	3.8%	47,468,013	1,780,050
Niger	0.32	3.3%	3,267,496	108,917
Malawi	0.24	2.5%	3,563,840	89,096

These results give testable predictive values for the number of individuals that suffer from endometriosis. It would therefore be possible to compare actual endometriosis population data with these data to test the accuracy of this model. At the time of writing there is only one publication that affords this opportunity.

2.5.1. Proof of Concept

A recent publication by Gylfason et al [212] reported the incidence of endometriosis in an Icelandic population over 20 years, this data allows the comparison of real world and predictive endometriosis prevalence. The population of Iceland is relatively homogenous, composing primarily of Nordic-Caucasians. The authors noted that women participating in this study originated mainly from the urbanised capital ensuring similar living conditions, diet and standard of living [212], thus making it an ideal sample population to test the prediction model. Gylfason et al reported an incidence of 1,303 women diagnosed with endometriosis, in the age group 15-49, between 1981-2000. The

National Statistical Institute of Iceland [213] gives the female population for this age group and date range to be 15,325 giving an endometriosis incidence rate of 9.0% over 20 years. To test the model the predicted ERI score needs to be converted into a percentage of population. The United Kingdom has a predicted ERI score of 0.96, and a known endometriosis prevalence of 10.0%. It is therefore reasonable to conclude that an ERI score of 0.96 is equal to a prevalence rate of 10%. Therefore, by decreasing the % prevalence proportionally to the ERI we can predict the % prevalence in a country. Using this method predictive and known % prevalence for endometriosis can be compared based on the data for Iceland.

The ERI score for Iceland was 0.92 equating to a reduction of 4% on the 0.96 ERI for the UK. If we proportionally decrease the % prevalence we arrive at a predicted percentage prevalence of 9.6%. In terms of number of women with endometriosis this led to a predictive value of 1,469 women with endometriosis. Comparing this to the real world data recorded in Iceland gives an accuracy of 88.7% for the prediction model in this population.

2.5.2. Limitations

The conclusions that have been made in this chapter have limitations, such as the disparity between dates when certain data were collected. For example, the data for age at menarche in Britain was collected in 1986 whereas the Bangladesh data was collected for 1993. Due to the observed downward trend in age of menarche in developed countries over the last century [214], ideally it would be better to obtain data for age at menarche for the same time periods for each country.

The prediction of endometriosis prevalence is also limited by the risk factors that have been chosen. For example, each risk factor cannot be weighted equally i.e. parity is much more influential on the risk of developing endometriosis than ovarian cancer incidence, however presently there is

insufficient evidence to accurately weigh each risk factor. Therefore, without an objective method of weighting the risk factors it is impossible to correct for any weighting errors that may occur. Nevertheless, evidence collected so far affords a compelling argument for the variation in endometriosis prevalence throughout the world.

A further limitation may be the exclusion of certain risk factors due to lack of available data as mentioned in the discussion. For example, dioxin or other EDCs may play a significant role in the development of endometriosis, but with data on EDC exposure limited, it cannot be accounted for in this prediction model.

Additionally, this prediction model assumes that all populations are homogenous, however this is not the case. The USA and UK, for example, comprise a large diversity of ethnicities and cultures, all of which have differing levels of the risk factors used to create this model of prediction. For example, this model indicates that the UK would have a high prevalence of endometriosis based on the risk factors applied such as low parity and high alcohol consumption. However, the Muslim population of the UK would consume little to no alcohol and an orthodox Catholic family would be expected to have a high number of children. Hence, these populations within the UK would be expected to have a lower prevalence of endometriosis. Therefore, it must be taken into account that this model can only estimate the prevalence of endometriosis for the population of a country as a whole. The prevalence of endometriosis within sub-populations is likely to significantly vary.

Chapter 3. Of Epigenetics and Endometriosis

3.1. Background

Epigenetics is a relatively new field of science. Over the last 20 years in particular, scientific investigation into epigenetic mechanisms has expanded exponentially. Figure 23 shows the rapid increase in publications with the keyword 'epigenetics' in the title over the last 22 years according to the PubMed database.

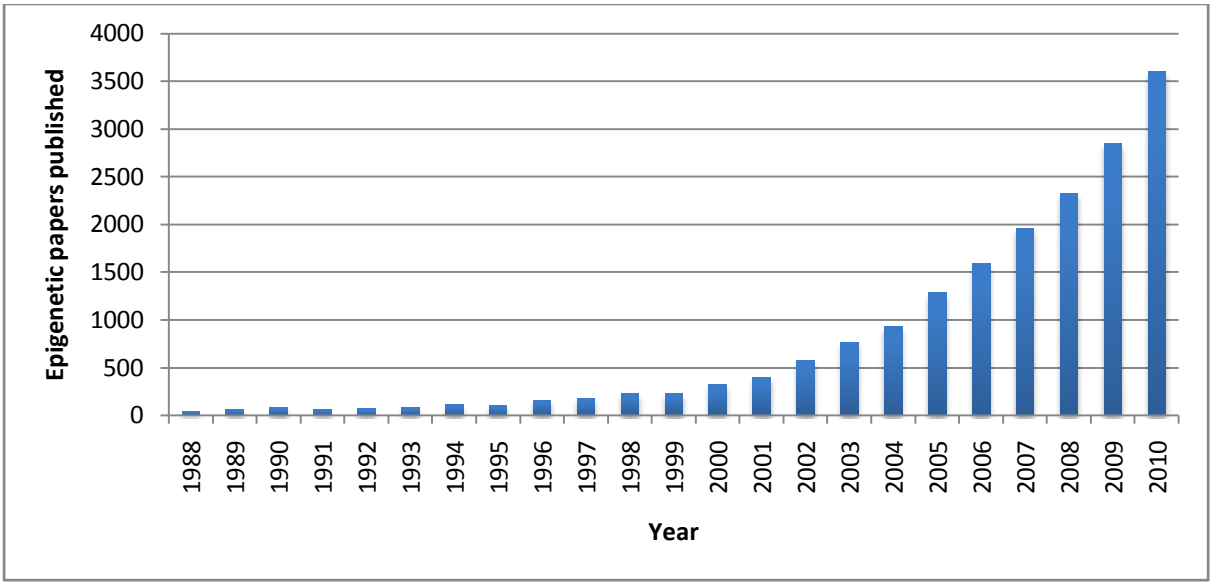


Figure 23. Epigenetic publications over the last 22 years

Epigenetics is defined as heritable changes in gene expression that are not associated with changes in the nucleotide sequence. More recently epigenetics has been used as an umbrella term for all processes, factors and interactions that alter transcriptional regulation or expression of a gene without changing the DNA code, and many describe it as an additional 'layer' of transcriptional regulation. Any cell within the body contains the same genetic information, however individual cell types display a wide variety of functions and morphology. Epigenetic mechanisms allow cells to be 'programmed' to perform specific functions and specialised by regulating the genetic expression of information. For many years the *Zeitgeist* among geneticists was that heritable change can only be mediated via a change in nucleotide sequence. Epigenetics has changed this preconception by

showing that it is not just the sequence of DNA, but the marks it carries that can result in heritable phenotypic changes. Recently there have been several publications concerning the involvement of epigenetic mechanisms in endometriosis [69], this chapter will explore and build upon this current knowledge.

3.1.1 DNA Methylation

Methylation is perhaps one of the most widely studied epigenetic marks. A methyl group can be bound and removed from the 5' carbon of a cytosine on a CpG dinucleotide in DNA. If a region of DNA contains a large portion of CpG dinucleotides it is referred to as a CpG island. Methylation controls the expression of genes by altering the structure of chromatin. Chromatin is comprised of DNA wrapped around a core of eight histone proteins (two sets of H2A, H2B, H3, H4 which can also be methylated), this together is known as a nucleosome [215]. The packing of these nucleosomes defines the structure and nature of chromatin. Active chromatin (euchromatin) is characterised by low levels of histone and DNA methylation and high levels of histone acetylation and has an open structure allowing access of transcription factors and polymerase enzymes. Tightly packed chromatin (heterochromatin) is characterised by highly methylated histones and DNA and low levels of acetylation and is transcriptionally inactive (Figure 24). If the CpG dinucleotides are methylated in a particular gene (usually in the promoter region) [216], the gene may become silenced or down regulated [217, 218]. Conversely, if the CpG islands in a gene promoter remain unmethylated, the gene will be more highly expressed [217, 218]. A significantly high percentage of all the CpG islands in the genome in any one cell type, at any one time display high levels of methylation, with estimates of 60-90% of all CpG residues being methylated [219]. These modifications are reflective of the fact that, in any one cell type, only tissue specific genes need to be active. Activation of the wrong gene in the wrong cell type may lead to the onset of diseases such as cancer or the development of other fatal abnormalities. The only exceptions to suppression by methylation are 'housekeeping' genes, which are essential for generic cell functions and are therefore constituently

activated in the majority of cells. The DNA of these housekeeping genes is mostly hypomethylated [220], reflecting their need for consistent activation.

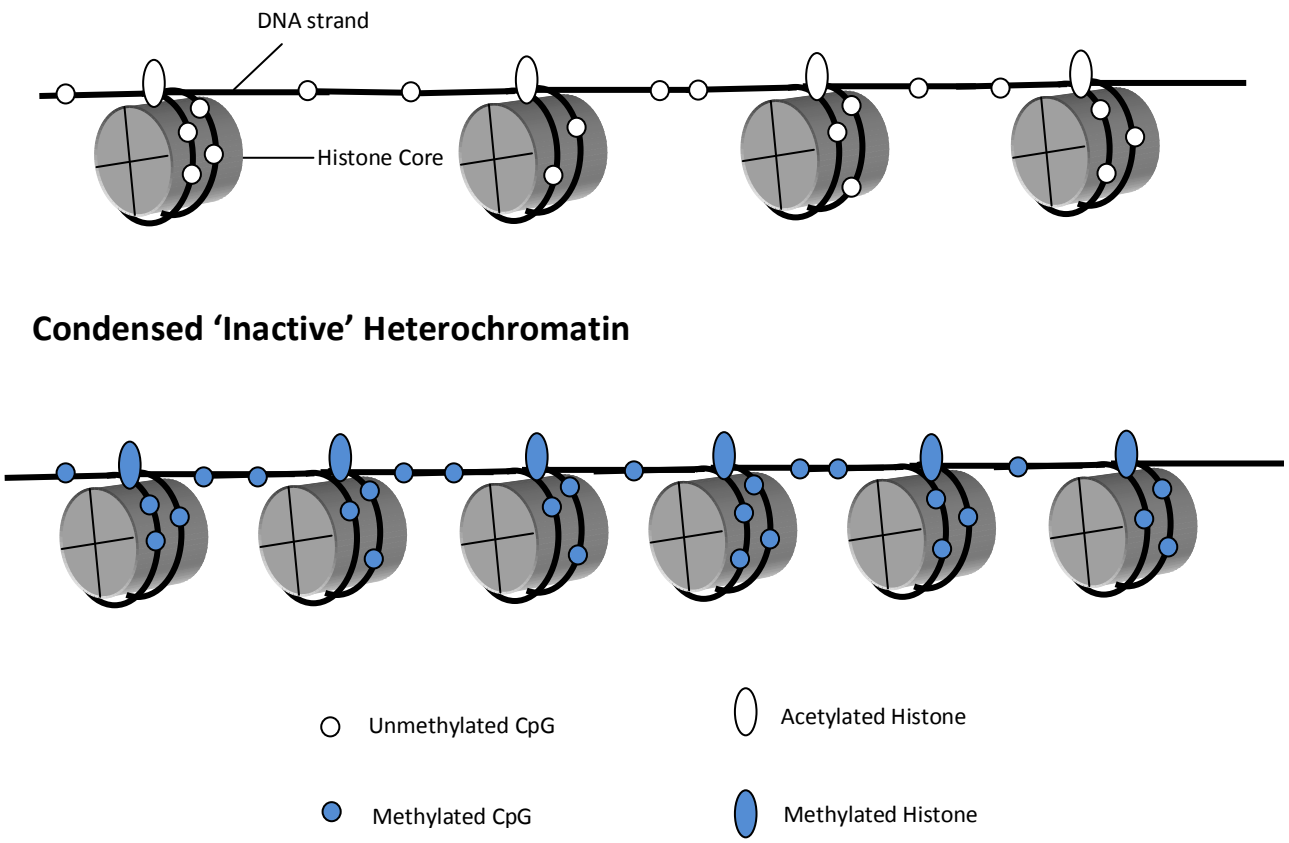


Figure 24. The relationship between histone and DNA methylation and chromatin structure.
Transcriptionally active Euchromatin, shown above, displays an open structure, the CpG islands in the DNA are unmethylated and the histones are acetylated. Heterochromatin displays a more condensed and transcriptionally inactive structure with methylated CpG islands and histones. Adapted from *Epigenetics* by J.Tost (2008) Caister Academic Press

The establishment of DNA methylation patterns is an active process, which is applied and maintained by DNA methyltransferase (DNMT) enzymes of which, there are three main isozymes in mammals, DNMT1, DNMT3a and DNMT3b. The universal methyl donor S-adenosyl-L-methionine (SAM) is required by the DNMT enzymes for the transfer of a methyl group to the C5-position on a cytosine residue. It is now recognised that the reaction is a Michael addition, whereby the Pro-Cys dipeptide of motif IV is the cysteinyl thiolate that covalently binds the C6 position of cytosine. A glutamyl residue of conserved motif VI protonates cytosine N3, which generates a reactive cytosine 4,5 enamine. This enamine attacks the sulphonium-linked methyl group of SAM. The methyl group is

transferred and a proton is removed from C5 resulting in the reformation of the double bond between C5 and C6. This results in the release of the enzyme by β elimination [221-223]. DNMT1 is responsible for copying and maintaining the existing pattern of methylation during cell replication [224], DNMT3a and DNMT3b introduce new methylation patterns and are thus known as the *de novo* methyltransferases [225], and recently DNMT2 and DNMT3L have been identified. Although the exact function of DNMT2 has yet to be fully characterised, some evidence suggests it has weak methyltransferase activity [226], not only for DNA but also for tRNA [227], however what biological function this serves has yet to be elucidated. DNMT3L appears to lack methyltransferase activity but evidence suggests it acts as an enhancer for DNMT3a and DNMT3b [228]. The pattern of DNA methylation laid down during gametogenesis and embryogenesis is essential for survival. Mice that have been engineered to show a dramatic loss of DNMT1 function presented with reduced weight at birth and rapidly developed aggressive T cell lymphoma [229]. Mouse knockouts for DNMT3a and DNMT3b proved similarly fatal [225] but with significantly earlier mortality than those with a single knockout.

It also appears that DNMTs function synergistically since disruption of DNMT3b only resulted in a 3% reduction in total global DNA methylation [230]. However, simultaneous disruption of DNMT1 and DNMT3b results in an almost total loss of methyltransferase activity and subsequent DNA methylation [230]. Further evidence for the co-activity between the DNMT enzymes was provided by Kim et at [231], who found that the N-terminus of DNMT3A and DNMT3B bind to the N-terminus of DNMT1 [231] and the co-expression of all three DNMT enzymes enhanced the activity of DNMT1 [231]. Collectively, this evidence suggests that full functionality and co-operation of DNMTs is essential for successful development and survival.

Recently it has been shown that DNMT1, DNMT3a and DNMT3b are over expressed in endometriotic tissue [232, 233]. These findings are likely to provoke new ideas regarding the origin and aetiology of endometriosis. For example, over-expression of these enzymes would be expected to alter global DNA expression in endometriotic cells. DNA microarray analysis of endometriotic tissue supports this expectation since a substantial number of genes display significantly altered expression patterns [234-239]. Another possible explanation for these observed irregularities in gene expression may be due to abnormalities in the regulation and function of the major transcription factor, NF-κB, in endometriosis (for review see Guo 2007 [240]). While this may be so, the introduction of epigenetics into the fray offers new insights into the origin and progression of disease, such as the combined effect of disrupting traditional and epigenetic regulators of transcription. In support of this notion Wren et al [241] demonstrated that epigenetic mechanisms such as histone modifications, methylation and acetylation may play a role in the aetiology of endometriosis. However, results from microarray studies provide an unusual paradox. The majority of studies found almost the same number of down regulated genes as there were up regulated in ectopic endometrial tissue. For example, Kao et al [235] reported 91 genes significantly over expressed and 115 under expressed. Similarly Eyster et al 2002 [237] and Eyster et al 2007 [234] reported more genes over expressed in ectopic endometrial tissue. Yet enhancement of DNMT function in endometriosis should lead to increased levels of DNA methylation hence, increasing the number of silenced or down regulated genes. This raises the question as to how a system can be in place where global gene expression in endometriotic cells should be down-regulated by over active methylation, and yet the evidence clearly shows many genes are up-regulated. However, it should be noted that Burney et al reported a higher frequency of under expressed genes in the eutopic endometrium of women with endometriosis vs disease free controls. A possible explanation to this paradox will be discussed in section 3.6. Of course it must be considered that not all genes are epigenetically regulated, genetic mechanisms are likely to play a significant role in aberrant gene expression in endometriosis.

It could be hypothesised that over-expression of DNMTs causes several key genes involved in regulating cell growth and apoptosis to be silenced or under-expressed, leading to the survival and proliferation of endometriotic cells. This is indeed the case for cancerous cells as demonstrated by Jacinto et al using HCT-116 colorectal cancer cells [242]. Current evidence may indicate a similar case for endometriosis, as endometrial cells from women with endometriosis display reduced apoptotic markers [243] and a reduction in the expression of pro-apoptotic genes [244] when compared with controls. Indeed, this apoptotic resistance is evident for many types of malignant cells where DNMTs are also frequently over-expressed [245].

Discovery of the over-expression of DNMTs in endometriosis may lead to new therapeutic regimens. DNMT inhibitors have already been developed for the treatment of cancer, with compounds such as 5-aza-2'-deoxycytidine proving effective at reducing tumour growth in human trials [246]. This anti-tumour effect is thought to be due to restoration of a previously hypermethylated tumour suppressor genes to its active state. This effect could potentially be exploited for the treatment of endometriosis by restoring both the expression of the hypermethylated progesterone receptor [247] and the pro-apoptotic actions of the p53 gene, which is reportedly down regulated to near undetectable levels in endometriotic cells [248]. Despite the benefits promised by these new treatments, concerns regarding the use of such drugs were raised. Since they activate tumour suppressor genes, they could also potentially activate proto-oncogenes, counter-intuitively increasing tumour occurrence. Fortunately, evidence suggests that therapeutic use of demethylating agents does not appear to increase tumour incidence [249], although far more evidence is needed before these drugs can be declared safe for mass use. The potential application of these drugs for the treatment of endometriosis, given the evidence provided, is therefore worthy of further investigation.

3.1.2 Epigenetic Modification of Steroid Synthesis and Receptors in Endometriosis

The deregulation of DNMTs is not the only evidence supporting the hypothesis that epigenetics plays a major role in endometriosis. Izawa et al [250] demonstrated that the expression of the cytochrome p450 aromatase enzyme (CYP19) is dependent on the methylation status of its promoter by treating endometriotic cells with the demthylating agent 5-aza-deoxycytidine and observing the fold change in aromatase mRNA expression. Current studies have reported either a weak or no association between polymorphisms of the aromatase gene and endometriosis [251-253] that could account for the observed over expression of aromatase in endometriosis. Those studies that have associated certain aromatase polymorphisms with endometriosis have been criticised for faulty data analysis or non reproducible results [254].

Aromatase is a key enzyme involved in the synthesis of estrogen and plays a crucial role in the pathogenesis of endometriosis. With the exception of two studies [255, 256] aromatase is reported to be highly up regulated in endometriotic cells whilst being nearly undetectable in normal endometrium [106, 257]. The importance of aromatase in the pathology of endometriosis is aptly demonstrated by the use of aromatase suppressing drugs for the treatment of the disease. This class of drugs, although with limited clinical data, have shown to be effective in the symptomatic treatment of endometriosis [122-124]. Aromatase is normally expressed in a cyclic fashion throughout the menstrual cycle in eutopic endometrium however, expression levels are consistently elevated in endometriotic cells [258]. If the over-expression is initiated by hypomethylation of the promoter [250] and maintained by aromatase activating cytokines such as IL-6, IL-11 and TNFα, all of which have been shown to be dysregulated in endometriosis [39, 257, 259], a consequence would be over-expression of aromatase leading to increased synthesis of estrone, which is converted to estradiol, a potent estrogenic factor that initiates a number of pathways leading to the proliferation and survival of endometriotic cells [114]. The conversion of estrone to estradiol is catalysed by the

enzyme 17β-hydroxysteroid dehydrogenase type 1 (17βHSD I), which is reportedly up regulated in endometriosis [260]. The increased activation of aromatase in endometriotic cells leads to a self sustaining positive feedback loop for estradiol production, whereby prostaglandin E2 (PGE_2) activity induces the up regulation of aromatase [258] leading to increased estradiol levels. In turn, this leads to the up regulation of the cycloxygenase-2 enzyme (COX-2) [261] resulting in the formation of more PGE_2, an important factor in the pathology of endometriosis [262] thus the cycle becomes self perpetuating (Figure 25).

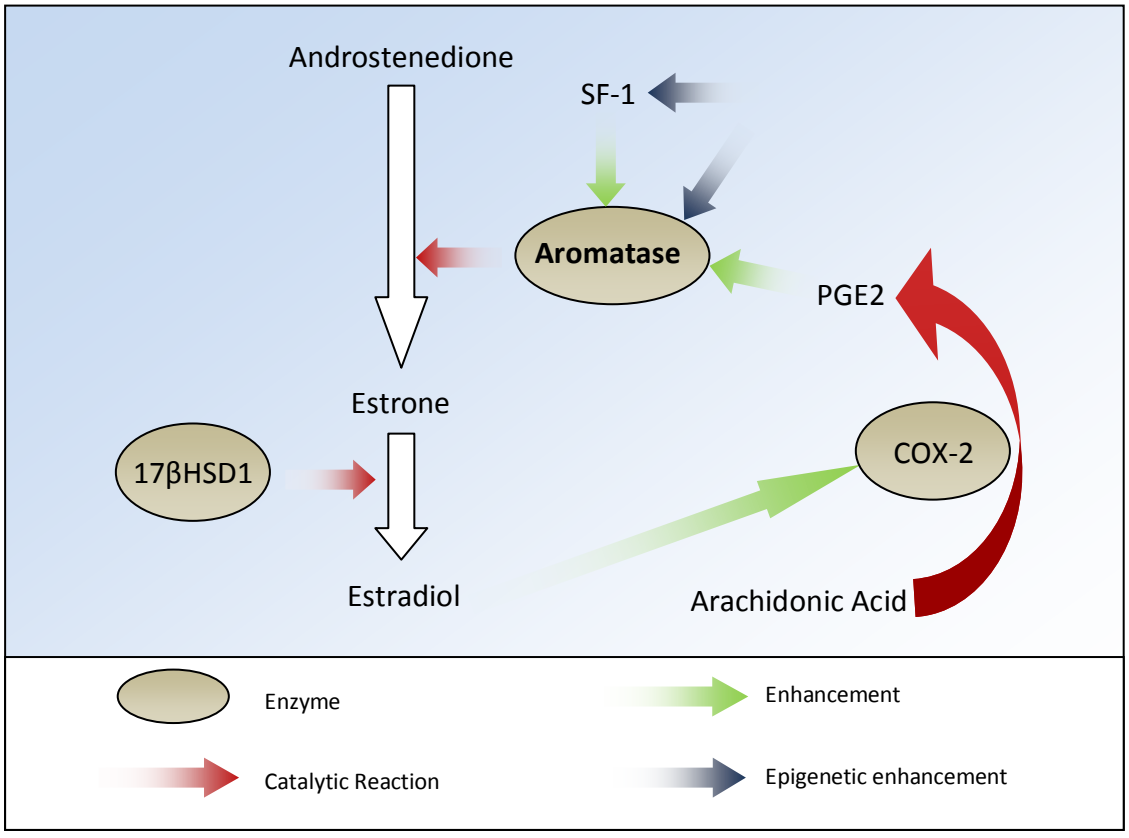


Figure 25. The aromatase cycle.
Adapted from; *Endometriosis in Clinical Practice*, by David Olive (2005) Taylor and Francis

Aberrant methylation of the aromatase promoter is not the only factor altering gene expression due to epigenetic alterations in endometriosis. Regulation of aromatase is mediated by steroidogenic factor-1 (SF-1) which is the aromatase enhancer, and chicken ovalbumin upstream promoter transcription factor (COUP-TF) [114] which is its repressor. Indeed, SF-1 has recently been shown to

be over-expressed in endometriotic cells [263]. An explanation for the apparent over-expression of SF-1 has been proposed by Xue et al [264] who observed hypomethylation of the CpG island near to its promoter region. The discovery of epigenetic modifications in the promoters of aromatase and its enhancer SF-1 provide some understanding of the establishment of the reported positive feedback loop. As with mutations, once these epimutations are established, they are retained throughout each cellular division, ensuring the survival of the endometriotic cells.

It is not only the synthesis of estrogen that is affected by aberrant methylation in endometriosis. In order for estrogen to mediate its mitogenic effects within the cell it must first bind to its receptor, of which there are two variants, estrogen receptor α (ERA) and estrogen receptor β (ERB) coded for by separate genes. Cells that over express estrogen receptors are highly sensitive to estrogenic effects. Such cell types include breast and ovarian cancer cells [265, 266] which, as with endometriotic cells, possess an enhanced proliferative capacity. Recent studies have shown that the mRNA of one isoforms of the estrogen receptor (estrogen receptor 2 gene, encoding estrogen receptor β) is over expressed in endometriosis [267]. This apparent over-expression was found to result from hypomethylation of the CpG islands in the promoter of the ESR2 gene. ERB is important as it is known to regulate several genes involved in signal transduction, cell cycle progression and apoptosis [268], however in contrast to endometriosis several studies have shown ERB to be down regulated in ovarian cancer [269, 270] and it thought that loss of ERB expression may induce malignant transformation [271]. It is also important to note that several endocrine disrupters though to be risk factors for endometriosis mediate signalling cascades via ERB [272, 273]. Therefore, not only does epigenetic modification lead to enhanced estrogen production but it also leads to increased sensitivity towards estrogen and estrogen-like compounds in endometriotic cells, resulting in a self sustaining endometriotic cell population.

Due to abnormal estrogen synthesis and metabolism observed in endometriosis, progestogenic agents are commonly administered to women with the disease in order to suppress endometriotic cellular proliferation by down regulating estrogen production and acting as an anti-inflammatory agent [274]. The efficacy of progestogens in relieving persistent pain symptoms associated with endometriosis is relatively poor [275], and a reported 9% of women are completely unresponsive to progestogen treatment [118]. The relative inefficiency or total lack of response to progestogenic treatment has thus, led some to conclude that endometriotic cells are somehow resistant to the effects of progesterone. Evidence for this comes from studies of the progesterone receptors in endometriotic cells. As with ER, there are two progesterone receptor isoforms, PR-A and PR-B. Unlike the ER, these encode as splice variants of the same gene and each has distinct functions and distinct levels of expression in the eutopic endometrium, depending on the phase of the menstrual cycle. PR-B is a transcriptional activator for several genes containing a PR-B dependent promoter. PR-A, on the other hand is a transcriptional repressor for PR-B and ERα [276]. Studies have shown that PR-B expression is absent, and only very low levels of PR-A are expressed in endometriotic cells [110], offering some explanation for progesterone resistance in endometriosis. Additionally, Wu et al [247] showed that the aberrant hypermethylation of the PR-B promoter, reduces its expression to an almost silenced state. PR-A and PR-B, although coded by the same gene, have distinct promoters, thus it may be possible that aberrant methylation of the PR-A promoter may be responsible for its reduced expression. Conversely Wu et al suggested that alteration of PR-A expression may not be due to altered methylation of its promoter, but is likely due to other as yet, unknown mechanisms. Nevertheless, it is reasonable to conclude that there are epigenetic mechanisms by which estrogen production is both enhanced and unopposed in endometriosis (Figure 26).

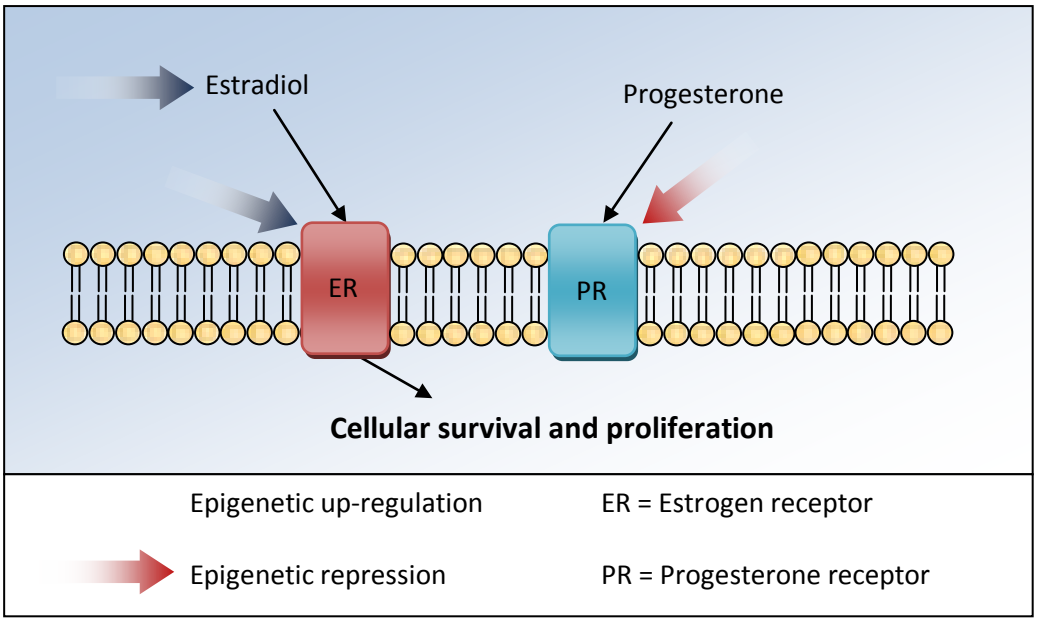


Figure 26. Epigenetic enhancement of estrogen sensitivity in endometriosis

3.1.3 HOXA10 in Endometriosis

HOX are a family of genes containing homeobox domains that act as transcription factors essential for regulating genes associated embryonic development. Several members of the HOX gene family play crucial roles during embryogenesis [277], for example HOXA9, HOXA10, HOXA11 and HOXA13 are involved with the development of the female reproductive tract, and unlike the majority of HOX genes they are expressed into adulthood [278]. A novel feature of the HOX genes is that the positional location of the gene along the chromosome is related to the positional location of the organ for which it is responsible (Figure 27). The involvement of HOXA10 in the development of the uterus affords specific interest with regards to endometriosis since any aberrant expression of HOXA10 may result in abnormalities, in either the function or morphology of the uterus. HOXA10 expression is reportedly down regulated in patients with endometriosis [279, 280], perhaps reflecting the findings that patients with endometriosis are more likely to present with anatomical complications of the reproductive tract [281, 282]. HOXA10 under-expression in endometriosis patients may also explain the associated subfertility observed in these patients since HOXA10 along with HOXA11 are responsible for successful implantation of the embryo [283, 284].

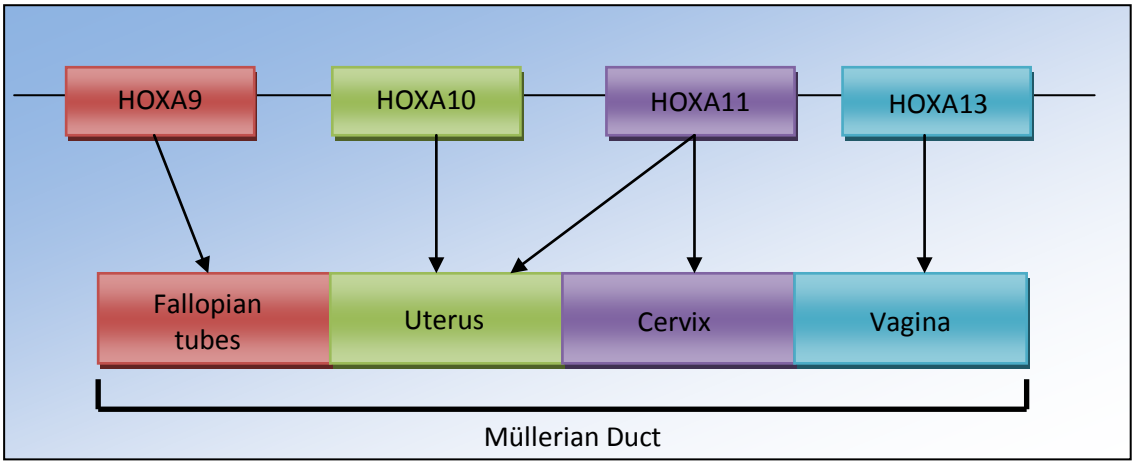


Figure 27. HOX genes in the development of the Müllerian duct.
Adapted from; *Endometriosis in Clinical Practice*, by David Olive (2005) Taylor and Francis

The origin of the down regulation of the HOXA10 gene in endometriosis was investigated by Wu et al 2005 [285] and Kim et al 2007 [286]. Wu et al's study screened eutopic endometrium of women with endometriosis, whereas Kim et al screened eutopic endometrium from baboons with experimentally induced endometriosis. Both studies identified hypermethylation in the promoter region of the HOXA10 gene. However, these studies examined only methylation patterns of HOXA10 in the eutopic endometrium of endometriosis cases and controls, but did not examine the methylation status of HOXA10 in ectopic endometrium. Wu et al's study was also confined only to women with stage III-IV endometriosis. Therefore, definite conclusions regarding the aberrant expression of HOXA10 in endometriotic tissue in humans cannot be made. However, a study by Lee et al 2009 [287] using a murine model of experimentally induced endometriosis, found inducing endometriosis led to methylation dependant changes in HOXA10 expression in *eutopic* endometrium. This essentially turns current thinking on its head, as it has long been thought eutopic endometrium dictates the fate and function of ectopic endometrium (via polyclonal origin of ectopic endometrium from refluxed eutopic cells [288]) not, as Lee et al demonstrated, the other way around. Although it may be that interplay of signalling exists between the two cell types, the mechanism by which ectopic endometrium can influence epigenetic alteration of eutopic endometrium remains to be elucidated.

Interestingly, further study has reported that HOXA10 hypomethylation can be induced by *in-utero* exposure to diethylstilbestrol (DES) [289], a known endocrine disruptor. The ramifications of DES exposure are discussed further in section 3.5. The effect of altered HOXA10 expression in eutopic endometrium is obvious in terms of uterine morphology and embryonic implantation. What the biological significance of HOXA10 down regulation, in endometriotic tissue may be remains speculative. Some clues may arise from microarray study of HOXA10 knockdown cells, which reported HOXA10 as a regulator of hundreds of genes involved in a variety of cellular processes [290]. Of particular interest was the finding that HOXA10 knockdown led to a 5.78 fold increase in the CYP19 (aromatase) gene, the significance of which is discussed in section 3.2. It is also important to note that HOXA10 and HOXA11 are progesterone responsive genes (the significance of altered progesterone signalling is discussed in Section 3.1.2) and members of the HOX family themselves regulate a number of other genes including IGFBP-1 (see Section 3.1.4) and integrins (see Section 3.1.7) [291]

Nevertheless, the altered expression of HOXA10 in women with endometriosis may provide an explanation for one of the most puzzling aspects of endometriosis, which is, if retrograde menstruation is near universal, why do only a fraction of women develop endometriosis? It may be that HOXA10 aberrations result in improper development of the uterus. This is supported by the observed uterine anatomical abnormalities in women with endometriosis such as increased frequencies of septate uterus [292] and the reported decreased elasticity of the reproductive organs [282]. Both aspects are thought to increase the volume of menstrual reflux in some women, overwhelming the immune system which is thus unable to remove all the refluxed endometrial cells.

3.1.4 Insulin-Like Growth Factors and Imprinting

The insulin like growth factors (IGFs) are of particular importance when considering endometriosis since they are responsible for mediating the proliferative function of the endometrium. The

availability of IGFs is regulated by their association with IGF binding proteins (IGFBP) and IGFBP proteases. IGF-1 and IGF-2 are potent mitogenic factors and importantly, their expression is regulated by progesterone and estrogen [293]. Studies assessing the expression of IGF-1 in endometriosis provide discordant results. Some authors report higher levels of IGF-1 in the PF of women with endometriosis [294, 295], whilst others report either decreased expression of IGF-1 mRNA [296] or no change in expression [297]. However, a recent study of gene expression revealed that IGF-2 is significantly over expressed in endometriotic tissue [298]. A further investigation, which aimed to assess the differences in IGF expression between the eutopic and ectopic endometrium of women with endometriosis revealed that the expression patterns of IGF1 and IGF-2 in the ectopic endometrium were not synchronous with expression patterns in the eutopic endometrium and that the levels of eutopic expression varied between women with and without endometriosis [299]. Previously in section 3.1.2 it has been discussed how estrogen levels can be increased in endometriotic tissue by the aromatase cycle, therefore it can be postulated that it is the increased level of estrogen that enhances expression of IGFs. However, an epigenetic mechanism that has been widely studied, may offer an alternative explanation for the over-expression of IGF-2 in endometriosis. This mechanism originates from studies of many different types of cancer [300] and is known as loss of imprinting (LOI). Imprinting refers to parent of origin specific allelic expression which means that only one copy of an imprinted gene (either the maternal or paternal) is expressed, and the other is silenced by methylation. In this case, for example IGF-2 is an imprinted gene that is normally only expressed from the paternal allele. LOI however cannot explain the aberrant expression of IGF-1 in endometriosis as it is not an imprinted gene. Imprinting can be lost by changes in the methylation status of an imprinted gene resulting in either both alleles being activated or silenced. The resulting gene product will be either over or under-expressed and occasionally, as in several cancers and possibly endometriosis, this abnormal expression can contribute to the molecular pathology of a disease, particularly when the gene is involved in cellular invasiveness or proliferation. In females it could be considered that an entire chromosome is imprinted due to the

inactivation of one copy of the X chromosome. Imprinted genes are therefore more fragile than others, as it only requires the activation or inactivation of one allele to alter the entire gene expression. LOI was first documented in Wilms tumour, a kidney tumour that predominately occurs in children [301]. LOI of the WT1 gene is known to be responsible for the development Wilms tumour. Interestingly, WT1 expression is reportedly down regulated in the eutopic endometrium of women with endometriosis [302], whether or not LOI is responsible for this down regulation warrants further investigation. It is worth considering that in Wilms tumour and other types of cancer, mutation is responsible for only 5% of cases where WT1 deregulation leads to disease presentation [300], and therefore LOI is likely the dominant means by which WT1 deregulation occurs. If this holds true for endometriosis then the argument for LOI of WT1 in women with endometriosis becomes very strong. Whether or not LOI is responsible for the differences in expression of IGF-2 in women with endometriosis also remains unclear however this hypothesis clearly warrants further exploration.

Further investigation into the significance of imprinted genes within the genome revealed that the distribution of imprinted genes is not as sporadic as was first expected. Imprinted genes have been found to be closely flanked by others, forming clusters of imprinted genes at certain points in the genome. This led to the discovery that imprinted gene clusters can be regulated by an essential control sequence element known as an imprinting control element, or imprinting control region (ICR). The ICR that regulates IGF-2 for example, known as the Beckwith Wiedemann Syndrome (BWS) region, is also responsible for the control of nine other imprinted genes [303]. ICRs rely on methylation for control of repression or expression. IGF-2 for example, relies on methylation of the BWS ICR near the neighbouring gene H19 and is responsible for the selective IGF-2 expression on the paternal allele. The regulation of more than one imprinted gene by a single ICR has obvious implications in disease processes since one ICR alteration can produce knock on effects in many

genes. Since clusters of imprinted genes have differentially methylated ICRs [304], the control of imprinted genes is of great importance for understanding the aetiology of complex diseases such as endometriosis.

3.1.5 Epigenetics and the Environment

Epigenetics provides a link between genotype and the environment, and how exposures to different environmental, pharmacological and dietary elements can translate into heritable changes in gene expression. During early mammalian development the methylome is stripped and then re-applied in order to start development from a 'blank state' in which methylation errors are removed, for this reason it was thought that alteration of epigenetic marks such as methylation patterns could not effect subsequent generations. If epigenetic marks were heritable then the complex phenotypic consequences they encode, which may include disease phenotypes, must be heritable also. This leads to two issues that must be addressed before proceeding. Firstly, can epigenetic marks be altered by environmental influences and secondly, are these epimutations of the F^0 generation transmissible to the F^1 and F^2? In order to answer the first question it is important to consider that epigenetic marks are vulnerable to alteration during the demethylation events that occur early in development but are also venerable to change during each cell replication. DNA methylation by DNMTs is dependent on methyl donors such as S-adenosylmethionine (SAM), the major methyl donor, which is synthesised as part of the methionine cycle. The formation of this cycle is, in turn, dependent on dietary factors such as folic acid, vitamin B12, choline and betaine. Animal studies have shown that restricting dietary methyl donors produces a reduction in DNA methylation [305, 306], and in some cases, increased risk of developing tumours [307, 308], indicating that epigenetic aberrations mediated by dietary changes can result in complex disease phenotypes. The sparse epidemiological studies which have reported on the influence of diet and endometriosis [70] suggest that a diet high in fruit and green vegetables and low in meat and alcohol consumption is protective against developing endometriosis, however some findings were inconsistent [309]. From an

epigenetic perspective a diet high in fruit and green vegetables would provide a significant source of methyl donors, which may protect against demethylation induced genetic instability during foetal development [310, 311]. Alcohol consumption has been shown to alter histone acetylation and methylation patterns [312] which may account for the observed estrogenic effect of alcohol [313],

There is also the effect of synthetic environmental toxicants on epigenetic states to take into consideration. For many years the involvement of dioxins in the pathophysiology of endometriosis has been debated as dioxin acts as an endocrine disruptor. By binding to, and activating the Aryl Hydrocarbon receptor (AhR), dioxin causes the transcriptional activation of genes involved in estrogen synthesis and this action [314] is thought to promote the proliferation of endometriotic cells. In particular it is important to note that there is significant cross talk between the AhR and the estrogen receptors [315, 316]; see section 3.1.2 for the significance of altering estrogen receptor expression in endometriosis. Evidence for the role of dioxin in endometriosis resulting from animal studies has shown that primates and rodents exposed to high levels of dioxin exhibit an increased incidence of endometriosis [75, 317]. The validity of these data has however, been questioned [78], since the animal and human data are not robust, with the sample size and data analysis being called into question. A further criticism of the evidence is that only the effect on adults directly exposed to dioxin was taken into consideration, and that the incidence of endometriosis among their offspring was not reported. Nevertheless, evidence for the association between environmental toxin exposure and endometriosis continues to be an area of significant interest, with recent investigation focussing on the effect of body burden of organochlorides and their potential association with endometriosis. Several of these studies concluded that women with higher levels of organochloride exposure were not conferred an increased risk of developing endometriosis [318], while others demonstrated that women with endometriosis displayed higher serum levels of dioxin than controls [81, 82]. Thus, the implication of dioxin or other exogenous compound exposure in the development of endometriosis is sound in theory, but appears to lack definitive evidence. In particular, geographical areas where dioxin exposures have been high have reported no statistically significant increase in endometriosis

incidence. With regards to epigenetics, dioxin has been shown to alter the methylation status of the imprinted genes IGF-2 and H19 in mouse embryos [319] and therefore, may also alter the methylation status of other genes. However, the mechanism by which dioxin mediates this effect on methylation is unknown. This epigenetic alteration is maintained throughout gestation and into adulthood, demonstrating that dioxin exposure can have detrimental effects on the epigenetic status that may be heritable. However, further studies are needed to fully assess the effects of dioxin on epigenetic marks and the implications that any alterations may have in the pathophysiology of endometriosis.

It is not just exposure to environmental toxicants during adulthood that requires consideration but also exposure *in-utero*. Missmer et al [3] showed that *in-utero* human exposure to diethylstilbestrol (DES) increased the risk of endometriosis by 80%, but small sample size has been cited as a limitation of this study. DES is a synthetic estrogen which was commonly used as an anti-miscarriage drug between 1947 and 1971 [320]. Further human exposure resulted from the supplementation of DES in cattle feed as a growth enhancer [321]. DES has previously been documented to cause uterine abnormalities in the offspring of women and animals exposed to *in-utero* [320, 322]. Additional studies implicate early exposure to DES in a range of reproductive disorders and cancer [323]. Exposure to DES reportedly increases the expression of DNMTs, which are known to be over expressed in patients with endometriosis [324] and the reported epigenetic alteration of HOXA10 expression by DES has previously been discussed in section 3.1.3. Whether the over-expression of DNMTs in endometriotic tissue is the result of exposure to DES or other exogenous compounds is not certain. The preceding information provides substantiation for the hypothesis that dietary and environmental factors influence epigenetic states and may subsequently lead to disease presentation.

In order to complete the picture, the evidence for inducible epimutations being transmitted to the F^1, F^2 and beyond must be also be addressed. Several studies using rodents have hinted that complex disease traits can be transmitted to the F^2 via non-genomic pathways due to exogenous compound exposure of the F^0 [325-327]. Further study into such phenomena has revealed that the tumorogenic effects of DES are heritable in mice and humans [328, 329], possibly as far as the F^2 [330, 331], mediated by alteration of proto-oncogene methylation, possibly providing an explanation for transmissible, DES induced endometriosis through family lines. Bruner-Tran et al [332] demonstrated that early exposure to dioxin during mouse development led to alteration of methylation patterns which subsequently led to the mice developing an endometriosis like phenotype. However, it was not stated whether this phenotype was present in the F^1 or F^2 of exposed mice. Dietary habits of the F^0 have also been shown to affect epigenetic changes in subsequent generations. Initially, rodent studies demonstrated that maternal dietary restriction of methyl donors can translate to aberrant expression of several genes mediated by methylation in the F^1 [333-336]. Historical occurrence of mass dietary restriction in human females in the Netherlands, during 1944-1945 famine, resulted in reduced birth weight and increased insulin resistance of the offspring [337, 338]. Conversely, male dietary abundance in Sweden in the late 19th century led to increased mortality due to diabetes in their paternal grandchildren [339]. Due to the transgenerational effects of diet, further investigation is therefore warranted into the dietary habits of the parents and grandparents of women with endometriosis.

Due to the complex nature of endometriosis, diet alone is unlikely to be the main factor when considering the epigenetic involvement in endometriosis. However, the involvement that diet plays in the alteration of epigenetic states in endometriosis is a field worthy of study given the evidence that diet can influence deleterious epigenetic modifications and possibly contribute to the development of cancer [306].

The evidence presented here leads to the tantalising possibility that endometriosis can originate from epigenetic and hormonal modifications resulting from *in-utero* exposure to environmental estrogenic substances such as dioxin and DES, and furthermore, the epigenetic aberrations and therefore the disease itself becomes heritable. Further evidence is provided by a study that reported the effect of foetal and neonatal exposure to the endocrine disruptor Methoxychlor and its metabolites. This study concluded that early developmental exposure to these endocrine disruptors led to permanent alteration of the methylation states of the ESR2 promoter via up regulation of DNMT3B activity, ultimately leading to alteration of ovarian steroid production [272]. Thus, providing further evidence that endocrine disruptor exposure from environmental sources can lead to permanent epigenetic alterations that favour the development of endometriosis. This epigenetic hypothesis can link together many of the characteristics observed in endometriosis. In particular, the way in which endometriosis displays heritability, but can also arise with no family history. However, this is a double edged sword since not only can exogenous estrogens be harmful, they can also be beneficial. Studies of the plant isoflavone genistein have shown that it has protective effects against endometriosis by reducing the expression of ERα and increases expression of progesterone receptors [340]. A further investigation revealed that supplementation with genistein resulted in regression of endometriotic implants in animal models [188]. Therefore, the use of compounds analogous to genistein as a possible treatment for endometriosis should be considered.

3.1.6 Micro-RNAs and Endometriosis

Micro-RNAs (miRNA) are a class of small non-coding RNAs ~22 nucleotides in length. Since their initial discovery, over 500 individual miRNAs have been identified in the human genome [341] and more continue to be characterised. MiRNAs are included as an epigenetic mechanism since they possess the ability to regulate the expression of genes without altering DNA sequence. However, unlike the transcriptional repression action of DNA methylation, miRNAs act post-transcriptionally by binding to target sequences of mRNA. The way in which miRNA functions as a repressor is by

possessing a complimentary sequence to a specific protein coding mRNA. Upon binding to its target sequence the miRNA can interfere with the binding of polymerase or recruit endonucleases to break down the target mRNA (Figure 28).

Regulation of genomic expression by miRNA is essential for development and survival [342]. This is not surprising considering it is estimated that around 30% of protein coding genes are regulated by miRNAs [343]. MiRNAs regulate a wide range genes involved in cellular functions such as proliferation, apoptosis, differentiation, development and regulation of the immune system [344-346]. Regulation of miRNA expression is a finely controlled mechanism involving much the same transcriptional machinery as is involved in protein coding gene expression. There are several instances of miRNAs forming double negative feedback loops with regulators of miRNA transcription [347-351] adding an autoregulatory element to their expression. Recently however, it has been reported that miRNA genes can be epigenetically regulated themselves, by DNA methylation [352-354]. In particular a study by Saito et al [355] demonstrated that treatment of T24 bladder cancer cells with a combination of demethylating agent 5-aza-2'-deoxycytidine and histone deacetylase inhibitor, 4-phenylbutyric acid, led to ~5% of human miRNAs up regulated more than 3 fold. It is also worth noting that some miRNAs have tissue and developmental stage specific expression patterns [343] meaning that endometriotic cells are likely to have a specific pattern of miRNA expression, possibly aiding diagnosis or characterisation of endometriotic cells. It is therefore, noteworthy of the importance of miRNAs, not only in maintaining cellular function but also the potential for miRNA deregulation in contributing to disease presentation.

Endometriosis has long been recognised as an immunological disease with the characteristics of chronic inflammation. Thus, it is important to consider the role of miRNAs in endometriosis due to

their regulation of normal immune function and their involvement in other inflammatory diseases [356]. At the time of writing there have been two well designed studies which analysed the expression of miRNAs in endometriosis. Pan et al 2007 [357] analysed the differential miRNA expression patterns of eutopic and ectopic endometrial cells. Analysis of 287 miRNAs detected in endometrial cells from women without endometriosis, paired eutopic, ectopic and unpaired ectopic endometrial cells identified 48 differentially expressed miRNAs in these tissues with a significant reduction in the levels of miRNA expression in disease tissue. This, in part, correlates with the finding that DNMTs are over expressed in ectopic endometrium, which may lead to hypermethylation of certain miRNA promoters, resulting in the observed reduction in their expression.

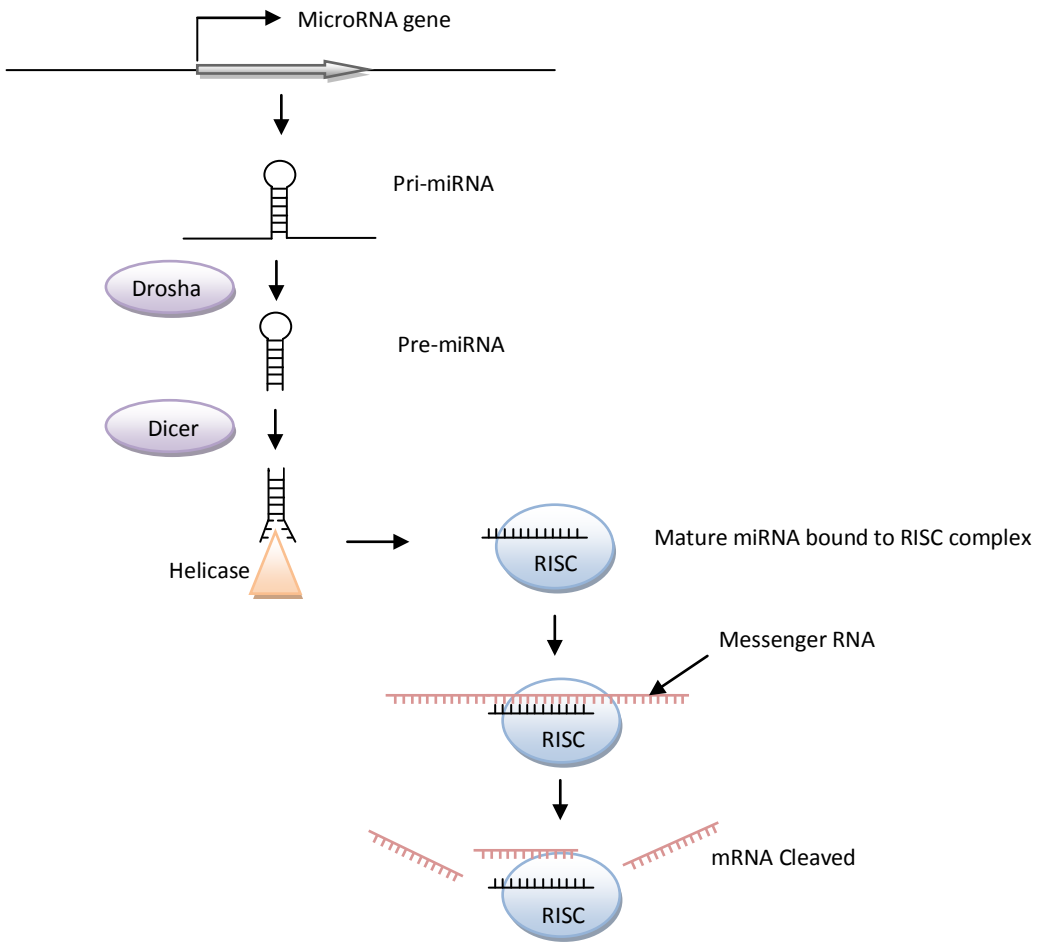


Figure 28. Biogenesis and mode of action of miRNAs.
Adapted from *Epigenetics* by J.Tost (2008) Caister Academic Press

The authors also observed that ovarian steroids have a mediating effect on miRNA expression but the mechanism by which this occurs, is however unknown. Ohlsson Teague et al [358] using a similar methodological approach to Pan et al analysed the miRNA expression patterns in paired ectopic and eutopic endometrial tissue and identified 22 miRNA differentially expressed in ectopic tissue. However there is an interesting discrepancy between the two studies. The study by Pan et al found that all of the 48 miRNA's aberrantly expressed in ectopic tissue were down regulated. Conversely Ohlsson Teague et al found, of the 22 miRNA's aberrantly expressed, only 8 were down regulated and 14 were up regulated. A possible explanation for these discordant results may arise from differences in methodology between the two studies. Firstly, Pan et al took tissue samples from disease free controls as well as paired eutopic and ectopic tissue. Ohlsson Teague et al did not take tissue samples from disease free women as controls, rather using paired eutopic and ectopic endometrium from the same woman. Secondly, Pan et al demonstrated that miRNA expression is regulated by ovarian steroids, therefore the menstrual phase in which the tissue samples were taken is likely to have a bearing on the miRNA signature of the tissue sample. Indeed Pan and Chengini reviewed the differential expression of miRNA's during the menstrual cycle [359]. Significantly, Pan et al's study took tissue samples from women in the early-mid secretory phase, whereas Ohlsson Teague et al used samples from women in the follicular phase and secretory phase. It is particularly important to note that several authors have demonstrated that estradiol is an inhibitor of certain miRNA expression [357, 360, 361]. Therefore, increased levels of estradiol in the secretory phase and lower levels of estradiol in the follicular phase may account for the lowered expression of miRNA's reported by Pan et al and higher expression reported by Ohlsson Teague et al respectively.

These conflicting reports however, present a conundrum. It has previously been discussed that DNMT3A is over expressed in endometriotic tissue as reported by Wu et al 2007. DNMT3A is predicted as a target for miR-29c, therefore the reported up regulation of miR-29c by Ohlsson

Teague et al would appear contradictory to the finding of over expression of DNMT3A. An explanation for this may arise from the hormonal regulation of miRNA expression. If estradiol is inhibitory to miR-29c expression as it is with other miRNA's it is then significant that the study by Wu et al took 23 out of 25 tissue samples from women during the proliferatory or secretory menstrual phases when circulating estradiol levels would be high, this in turn would lead to down regulation of miR-29c and allow for increased expression of DNMT3A.

It is therefore possible that over expressed DNMTs either reduces miRNA expression directly by altering the methylation pattern of their promoters, or via an indirect mechanism that modulates the production of ovarian steroids. This also provides an explanation for the paradox mentioned in section 3.1 whereby increased DNMT activity may lead to genome wide over-expression of different genes in endometriosis. MiRNA expression, like gene expression, is regulated by methylation of CpG dinucleotides. Therefore, enhanced DNMT activity would result in the hypermethylation of a number of genes including miRNA genes. If this process silences or reduces expression of various miRNAs, then the ability of miRNA to inhibit their target gene is hindered; in effect enhancing the function of DNMTs can silence the silencers. The singular and combined effect of the abnormal expression of both DNMTs and miRNAs in ectopic endometrial cells may also explain the results from microarray studies conducted using eutopic and ectopic endometrium, which showed hundreds of genes aberrantly expressed, contributing to the complex and multifactoral nature of endometriosis. What are therefore, the implications of disrupting miRNA in endometriosis?

3.1.7 Can Epigenetics explain the Malignant Potential of Endometriosis?

Despite being a benign condition, endometriosis shares many characteristics with cancer. Indeed some authors have shown that endometriosis, particularly of the ovary (endometrioma), is prone to malignant transformation [362-365] with normal lifetime risk of developing ovarian cancer increased from 1.5% to 5-10% in women with ovarian endometriosis (the pioneering Dr Sampson was the first to report such a case in 1925 [366]). However, increase in risk is relatively small and cases that

match the criteria for direct malignant transformation of endometriosis are rare [367]. Women with endometriosis are not considered to be more at risk from cancer in general but evidence is accumulating to suggest that women with endometriosis may be at an increased risk of specific malignancies, including ovarian cancer, melanoma [368, 369] and non-Hodgkin's lymphoma [370, 371], however not all studies are concordant. The main limiting factor for studies examining associations between endometriosis and malignancies is small sample size, which leads to larger margins for error when judging associations. In light of this the Swedish National Board of Health and Welfare began collecting detailed medical records for discharges in an inpatient register from 1964. From this data several cohort studies have been extrapolated. The first study analysed the records for 20,686 patients hospitalised for endometriosis from 1969 to 1983 [154] and linked them to the National Swedish Cancer Registry to detect subsequent incidences of cancer. Significant risk increases were found for breast cancer, ovarian cancer and hematopoietic malignancies (mainly representative of non-Hodgkin's lymphoma). In particular it appeared that risk of developing ovarian cancer was related to duration of an existing case of ovarian endometriosis. Expansion of this study [372] included 64,492 women and confirmed the previous studies findings with the further inclusion that women with endometriosis appeared to have a decreased risk of cervical cancer.

A possible explanation for malignant transformation of endometriosis could be that the acquisition of endometriosis relies on a stepwise accumulation of genetic and epigenetic aberrations, with only a few mutational steps separating endometriosis and malignancy (Figure 29).

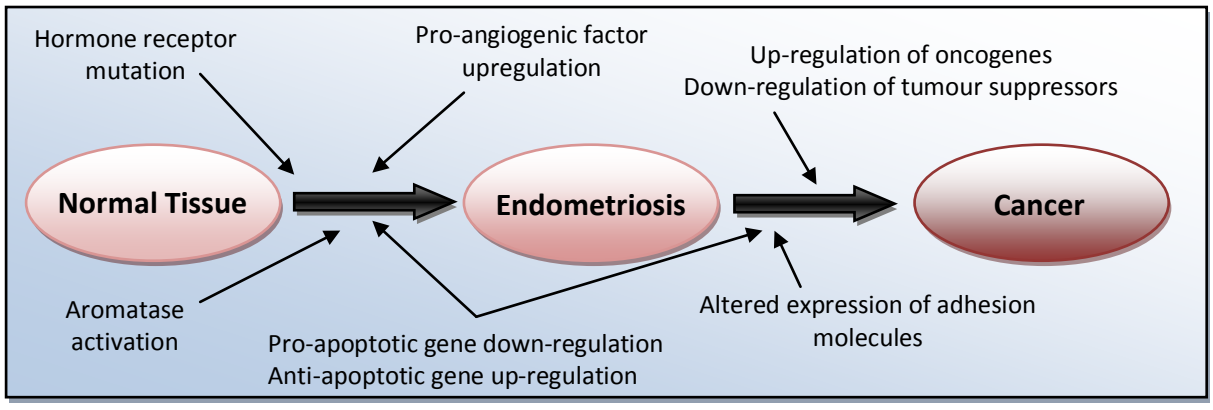


Figure 29. Stepwise malignant transformation of endometriosis

Chromosomal aberrations, such as loss of heterozygosisity (LOH) [373] and microsatellite instability [374] detected in endometriotic cells may indicate a genetically unstable state precluding them to malignant transformation. Studies indicate different types of endometriosis are more susceptible to malignant transformation. Ovarian endometriosis, for example, accounts for 75% of cases of tumorogenesis arising from endometriosis [375], this is supported by the finding of a higher frequency of chromosomal aberrations in ovarian endometriosis [376], suggesting it is more genetically unstable. Extraovarian endometriosis has been documented as undergoing malignant transformation but at a much rarer incidence [377]. Similarly, mutation leading to down regulation of tumour suppressor genes PTEN [378] and p53 [379] and over expression of oncogenes *c-myc, c-erbB-1 and c-erbB-2* [380] have been observed in endometriotic cells, which may increase the proliferatory nature of endometriotic cells and may also contribute to their malignant potential. An elegant study by Dinulescu et al demonstrated a stepwise progression from endometriosis to malignancy [381]. Female mice were engineered with an oncogenic allele of K-ras. These mice subsequently developed an endometriosis-like phenotype. Additional mutational deletion of PTEN induced a shift from an endometriosis-like phenotype to an ovarian endometrioid tumour, effectively demonstrating the transfer between disease states. Like endometriosis, over expression of DNMT's has been documented in a number of malignancies [382-386]. The over activity if DNMTs is thought to silence tumour suppressor genes, contributing to cancer progression [242, 387, 388]. This has been the basis for using demethylating agents for the treatment of cancer, which has led to some encouraging results [389, 390] and provides further evidence for the use of such agents in the treatment of endometriosis.

Endometriosis, like cancer, also displays metastatic potential. The expression of structural adhesion molecules such as cadherins are reported to be similar between endometriosis, ovarian endometrioma and borderline tumour [391]. Additional analysis of the expression of integrins,

another type of adhesion molecule, revealed that their expression was highly variable in endometriotic cells [392], whereas in normal endometrium their expression is tightly aligned with the phases of the menstrual cycle [392]. The abnormal expression of such molecules is thought to be an indicator of cellular dissemination and invasiveness [393] and is characteristic to both endometriotic and cancerous cells. In summary, there are several examples of epigenetic mechanisms being involved in the aetiology of endometriosis that need to be explored. The following studies will attempt to address and extrapolate novel data on these issues.

3.2 Aims

3.2.1. Insulin like Growth Factors and Imprinting

The aim of this study was to assess the potential for the involvement of LOI in endometriosis.

3.2.2. MicroRNAs and Endometriosis

The aim of this study is to analyse the miRNA data on endometriotic cells from the studies by Pan et al and Ohlsson Teague et al to elucidate possible implications of miRNA disruption in the aetiology of endometriosis.

3.2.3. Can Epigenetics explain the Malignant Potential of Endometriosis?

The aim of this study is to assess the possible involvement of epigenetic mechanisms in the malignant transformation of endometriosis. Similarities in miRNA patterns between the two diseases will be the main focus.

3.3 Methods

3.3.1 Insulin like Growth Factors and Imprinting

To date around 80-100 imprinted genes have been identified in humans and mice. A full database of these imprinted genes can be found at http://igc.otago.ac.nz/home.html [394]. In order to

investigate the possibility of any of these imprinted genes being disrupted in endometriosis this database of imprinted genes was cross referenced with microarray data. Microarray data was sourced from the PubMed citation database (accessed 12/08/2008) using the keywords 'endometriosis' and 'microarray'. This initial search yielded fifty results, which were narrowed to three articles with relevant information, Eyster et al 2002 [237], Eyster et al 2007 [234] and Kao et al 2003 [235]. All three studies used similar methods and obtained similar results. Further information on the function of any genes identified as being imprinted and disrupted in endometriosis was sourced from the Entrez Gene online database (accessed 21/02/2009).

3.3.2 MicroRNAs and Endometriosis

MicroRNAs disrupted in endometriosis were identified by searching the PubMed citation database (accessed 02/03/2009) using the keywords 'microRNA' and 'endometriosis'. At the time of writing this search yielded four articles, these results were narrowed to two articles with relevant information, Pan et al [357] and Ohlsson Teague et al [358]. Both studies used similar methods however results of these studies differed in some respects. In order to investigate the aim each miRNA identified as being differentially expressed in endometriotic tissue was investigated for potential targets by using the Sanger institutes microRNA target prediction software [395]. This software utilises the miRanda algorithm to identify potential binding sites for a given miRNA in genomic sequences. The algorithm uses a weighted scoring system and rewards complimentarity at the 5' region of the miRNA. Alignments where more than one base is mismatched at this region are discarded. Each potential target site in the 3' UTR is also checked to see whether the site is orthologous to transcripts from other species, each target must be conserved between at least two species. Any miRNA targets that matched with immunologic factors were searched for using the PubMed database using the keywords 'endometriosis' and 'name of immunologic factor'.

3.3.3 Can Epigenetics explain the Malignant Potential of Endometriosis?

Firstly the relationship between endometriosis and cancer was reviewed by searching the PubMed citation database using the keywords 'endometriosis' AND 'cancer' (accessed 29/04/2009). Secondly information was gathered on the involvement of epigenetic mechanisms in cancer and endometriosis by searching PubMed citation database (accessed 15/05/2009) with the keywords 'ovarian cancer epigenetic' and 'endometriosis epigenetic'. The relationship between miRNA expression patterns in endometriosis and cancer was investigated by cross referencing the miRNA data on endometriosis by Pan et al [359] and Ohlsson Teague et al [358] with data on the miRNA signature of cancer reviewed by Calin et al [396] and Corney et al [397].

3.4 Results

3.4.1 Insulin like growth factors and imprinting

The results of this analysis identified two imprinted genes: Ankryrin repeat and SOCS box containing 4 (ASB4) [398], and Brain-enriched guanylate kinase-associated protein (BEGAIN) [399] that were up regulated in endometriosis by 2.0 and 3.9 fold respectively.

The protein product of the ASB4 gene, located in chromosome 7q21, was identified as a member of the ankyrin repeat and SOCS box containing family. The SOCS box serves to couple suppressor of cytokine signalling (SOCS) proteins and their binding partners with the elongin B and C complex, possibly targeting them for degradation. The BEGAIN gene, located on chromosome 14q32.2, is reportedly a member of the NMDA receptor associated proteins which is involved in the assembly of neuronal synapses [400].

3.4.2 MicroRNA and Endometriosis

Pan et al demonstrated that several of the miRNAs deregulated in endometriotic tissue meditate the expression of estrogen and progesterone receptors. The epigenetic implications of this deregulation of ER and PR have previously been discussed in section 2.2. Pan et al also found that two of the miRNAs that mediate aromatase mRNA (miR-23a and miR-23b) down regulated in endometriotic cells. This could mean that not only does hypomethylation of the aromatase promoter increase gene transcription, but down regulation of these miRNAs could result in enhanced translation of aromatase mRNA transcripts. However, Pan and Ohlsson Teague did not thoroughly examine the inflammatory and immune systems under the control of miRNA that are implicit in the pathogenesis of endometriosis. During retrograde menstruation, the refluxed endometrial cells are normally cleared by the immune system. However, perturbations in the immune response observed in women with endometriosis are thought to provide a permissive environment in which viable endometrial cells are allowed to proliferate. Prospective immune system abnormalities include reduction in the efficiency of natural killer (NK) cells against ectopic endometrium [401] and diminished T cell proliferation [402]. It is interesting to note that of the 13 of the 48 significantly altered miRNAs identified by Pan et al and 2 of the 8 miRNAs identified as under expressed by Ohlsson Teague et al in ectopic endometrial cells, were involved in the differentiation of T cells [344], specifically CD8+ and CD4+ T cells. This provides a possibly explanation for the observed reduced number of activated CD8+ and CD4+ T-lymphocytes in the peritoneal fluid of women with endometriosis [403].

The effects of altered miRNAs expression may also have an indirect effect on T cell differentiation. Some authors have rightly pointed out that the aberrant expression of certain cytokines that regulate T cell and NK function are altered in endometriosis [404]. These cytokines such as IL-6, IL-8, IL-1, IL-1β, TNFα, INFγ, and TGF [39, 43, 405-408] are also regulated by miRNAs that are known to be

deregulated in endometriosis. These cytokines can be secreted from leukocytes and from endometriotic cells themselves. The miRNAs shown to be aberrantly expressed in endometriosis regulate a wide range of cytokines and growth factors thought to be involved in the immunobiology of endometriosis including those referred to above. For a summary of the factors regulated by miRNAs in endometriosis see Table 6.

Table 6. Micro-RNAs in Endometriosis

Gene	Regulatory miRNA	Expression in Endometriosis	Source	Consequence
VEGF	miR-126 miR-195	↓ ↓	Pan et al	Thought to be involved in the neovascularisation of endometriotic cells [409]
P450arom	miR-23a miR-23b	↓ ↓	Pan et al	Involved in the production of excess estradiol promoting the proliferation of endometriotic cells [410]
MMP-9	miR-191 miR-24	↓ ↓	Pan et al	Thought to be involved in the increased invasive capacity of endometriotic cells [411]
β-Catenin	miR-100	↑	Ohlsson Teague et al	
HOXA10	miR-29a miR-29c	↓ ↑	Pan et al Ohlsson Teague et al	Thought to be involved in endometriosis associated sub-fertility [412]
IL6	miR-126 miR-22 let-7i	↓ ↓ ↓	Pan et al	Alteration of the expression of these cytokines is thought to be responsible for the altered immune response in women with endometriosis, allowing a permissive environment for endometriotic cells [32]
IL-8	miR-145	↑	Ohlsson Teague et al	
TNFα	miR-125b miR-10b	↑ ↓	Ohlsson Teague et al Pan et al	
INFγ	miR-143 miR-125b miR-24 miR-29a miR-26b miR-27b	↓ ↓ ↓ ↓ ↓ ↓	Pan et al	
TGF	miR-23a	↓	Pan et al	A potent effector of peritoneal adhesion formation in endometriosis [413]
EGF	miR-29a	↓	Pan et al	Found to be significantly up regulated in late stage endometriosis, though to enhance proliferation of endometriotic cells [414]

3.4.3 Can Epigenetics explain the Malignant Potential of Endometriosis?

The overall genetic and epigenetic similarities between endometriosis and cancer are summarised in Table 7

Table 7. Similarities between endometriosis and cancer

	Endometriosis	Cancer
Monoclonal origin	Inconclusive	✓
Down regulation of p53	✓	✓
Down regulation of PTEN	✓	✓
Over expression of c-myc	✓	✓
Over expression of Bcl-2	✓	✓
Loss of heterozygosity	✓	✓
Chromosome 17 aneuploidy	✓	✓
Up regulation of VEGF	✓	✓
Over expression of CYP1A1	✓	✓
Altered microRNA profiles	✓	✓
DNMT over expression	✓	✓

MiRNAs can act as tumour suppressors and oncogenes. Table 8 shows the relationship between specific miRNA expression in endometriosis and different cancer types.

Table 8. Comparison of miRNA expression in cancer and endometriosis

miRNA	Expression in cancer (cancer type)	Expression in Endometriosis	Target gene/s	References
Potential tumour suppressors				
Let-7 (family)	↓ (lung)	↓	Ras	[415, 416]
miR-143	↓ (breast)	↓	ERK5	[417-419]
miR-145	↓ (colorectal, breast)	↓		
miR-195	↓ (various)	↓	Cyclin D1, CDK6, E2F3	[420]
miR-1	↓ (lung)	↑	MET	[421]
Potential oncogenes				
miR-21	↑ (various)	↓	PTEN	[422, 423]
miR-221	↑ (thyroid)	↓	KIT	[424]
miR-222	↑ (thyroid)	↓		

MiRNA 200a and 200b were identified as being down regulated in both endometriosis and ovarian clear cell and endometrioid cancer. Table 9 shows the targets of both genes and the function of each target.

Table 9. MicroRNA-200a and 200b targets potentially involved in malignant transformation of endometriosis

MiRNA-200a Targets		
Target name	**Function**	**Reference**
ZEB2	Over expression of ZEB2 associated with ovarian cancer progression, poor prognosis and metastasis	[425, 426]
KLF12	Involved in cellular proliferation and invasive potential of cancerous cells	[427]
MiRNA-200b Targets		
ZEB2	See above	[428]
ZEB1	Over expression leads to the progression of ovarian cancer	[429, 430]

3.5 Discussion

3.5.1 Insulin like Growth Factors and Imprinting

The discovery of only two imprinted genes dysregulated in endometriosis may not appear significant, however, when taken into consideration that LOI only a single gene is required to produce deleterious effects [431-433], the case for LOI in endometriosis gains support. The role of ASB4 and BEGAIN in the aetiology of endometriosis has yet to be clarified. However, the function of each of these gene products may provide clues as to their role in endometriosis. ASB4 has been reported be involved in vascular differentiation [434], a process that is very important in the establishment of endometriotic implant blood supply, therefore the aberrant expression of ASB4 may contribute to the vascularisation of endometriotic implants. More speculatively ASB4 has also been identified as a possible regulator of energy homeostasis [435]. Down regulation of this gene may therefore provide some explanation for the presentation of fatigue symptoms in women with endometriosis [436]. The influence of aberrant BEGAIN expression in endometriosis is more difficult to assess as this gene product is reportedly a member of the NMDA receptor associated proteins which is involved in the correct assembly of neuronal synapses [437]. Whether or not LOI is

responsible for the observed aberrant expression of imprinted genes ASB4, BEGAIN, WT1 or IGF-2 in endometriosis requires clarification, but it is an option that has never previously been considered.

Given the evidence from diseases where LOI is a causative factor, it is unlikely that LOI is a causative factor for endometriosis. For example, diseases characterised by LOI (e.g. Angelman syndrome, Prader-Willi syndrome) are usually also characterised by obvious phenotypic abnormalities from birth or in infancy [438, 439] resulting from LOI in either the germline or embryonic cells. It is more likely from the evidence presented here that LOI, if occurring in endometriosis, is similar to LOI in the pathogenesis of cancer, i.e. a contributory factor rather than a causative one, meaning that LOI would occur later in life in fully differentiated somatic cells, possibly by disruption of the methylation state of the ICRs by an as yet unidentified mechanism.

3.5.2 MicroRNA and Endometriosis

The involvement of different immunological, hormonal and environmental factors comprises a complex web of interactions that play a major part in the pathophysiology of endometriosis, allowing the survival, proliferation and vascularisation of endometriotic implants. Particularly important is the interplay between peritoneal macrophages and endometrial cells. Normally peritoneal macrophages (PM) suppress the proliferation and vascularisation of endometrial cells, however PM from women with endometriosis display a reverse effect, inducing endometrial cells to proliferate. The functions of PM are under the control of various cytokines and it has been postulated that aberrant expression of these cytokines and growth factors in endometriosis leads to altered PM function, creating a permissive environment in which endometrial cells can form endometriotic implants. For example, tumour necrosis factor (TNFα) is normally involved in the regulation of growth and shedding of the human endometrium and can enhance the production of prostaglandin by endometrial cells [440]. TNFα expression is also controlled by miRNAs (Table 6) and has been shown to promote inter-cellular adherence [40] possibly providing additional evidence as

to how refluxed endometrial cells attach themselves to the peritoneum. TNFα is a stimulatory factor for the production of Interleukin-8 (IL-8) and MMPs, which are thought to be involved in the invasion of endometriotic cells by increasing the adhesion of endometrial cells to fibronectin [441].

Estradiol up-regulates IL-1β and is essential for the development of endometriosis. Epigenetic alterations such as the induction of aromatase and increased expression of estrogen receptors can lead to increased estradiol production and sensitivity in endometriotic cells. The knock on effect that estradiol exerts over IL-1β expression is confirmed by significantly increased IL-1β concentrations in patients with endometriosis [442]. IL-1β induces the secretion of IL-8, which contributes to cellular invasiveness [443].

Estradiol is also known to up-regulate vascular endothelial growth factor (VEGF) [444], a potent angiogenic factor that is reportedly increased in patients with endometriosis [409, 445] and which is thought to regulate neovascularisation of endometriotic implants. VEGF expression is also under the control of miRNAs disrupted in endometriotic cells (Table 6). Not only are these cytokines involved in the survival, proliferation and vascularisation of endometriotic cells but contribute significantly towards the chronic pelvic pain associated with endometriosis, namely the formation of adhesions [446]. Adhesions can result from tissue damage caused by chronic inflammatory response or by surgery. IL-6 and TNFα in particular, have been identified as important for adhesion formation [447], both of which are under the control of miRNAs reported to be deregulated in endometriotic cells.

Defects in both the classical and epigenetic mediators of transcription may interplay, leading to two completely different phenotypes arising from identical genotypes, it could also lead to the enhanced or repressed expression of genes involved in the pathogenesis of endometriosis, (Figure 30). Pan et al and Ohlsson Teague et al have provided the first definitive evidence for the involvement of

aberrant miRNA expression in endometriosis. Some of the possible effects of such disruption are summarised in Table 6.

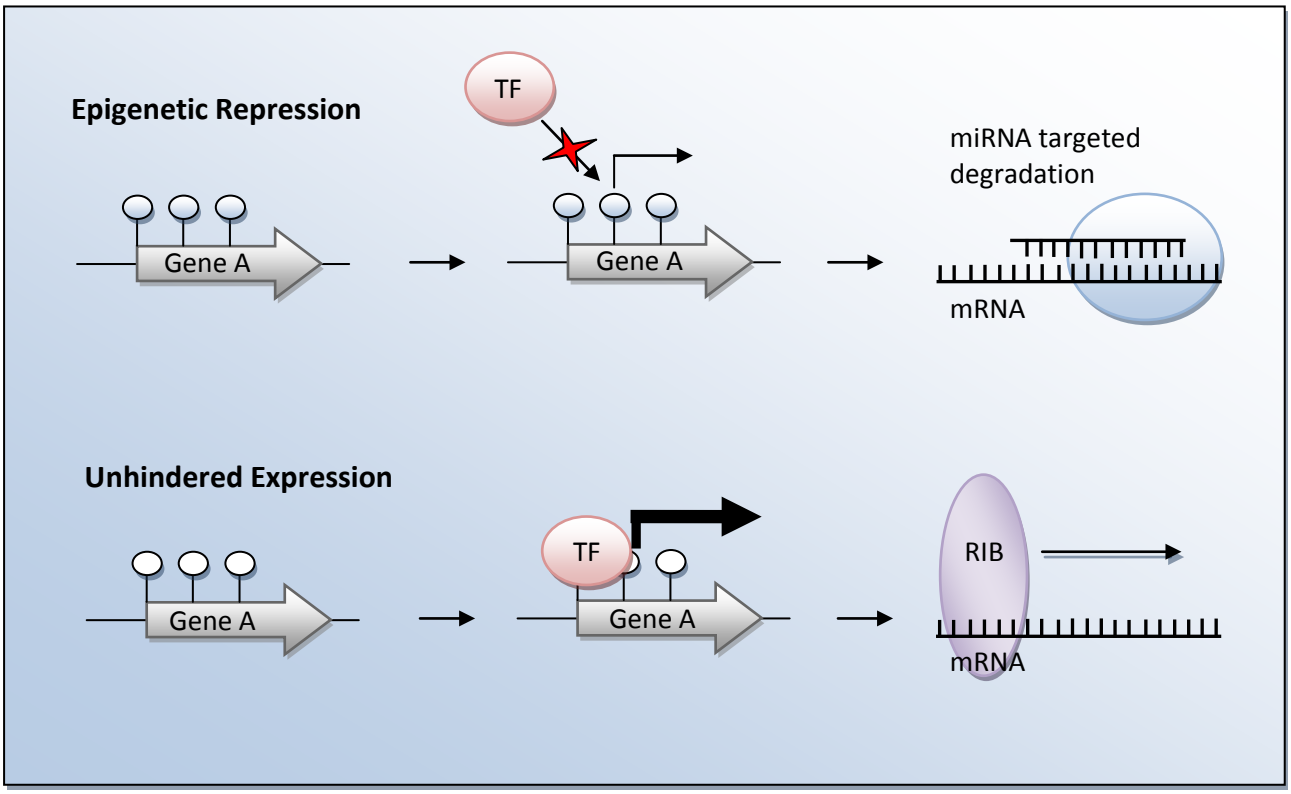


Figure 30. The effects of epigenetic mechanisms on gene expression

In one state Gene A displays hypermethylation patterns in the CpG islands of its promoter (represented by blue circles). This in turn prevents or reduces binding affinity of the classical mediators of transcription such as transcription factors (TF), thus lowering or halting transcription of the gene. If any mRNA is synthesised, microRNAs with a complimentary sequence may bind to it. This signals that the mRNA be cleaved by proteins such as an endonuclease preventing the translation of the mRNA by the ribosome (RIB). This results in either a great reduction or complete silencing of gene expression. In the other state, methylation of the CpG islands is not maintained, possibly by environmental influences such as exposure to exogenous compounds or a methyl donor deficient diet. Binding of transcription factors like NF-κB is therefore permitted, thus transcription of the gene is enhanced. Reduced expression of microRNAs results in the mRNA of gene A remaining uncleaved and therefore translation of the mRNA by the ribosome occurs with less hindrance. This deregulation of the epigenetic modifiers of gene expression in endometriosis could possibly be an explanation for the up and down regulation of some genes observed in endometriotic cells. This is only a hypothetical model, however if Gene A were a key gene in a process such as regulating the cell cycle or invasive capability it can be seen how endometriotic cells acquire their characteristics from epigenetic abnormalities. This also demonstrates how two very different expression patterns can be established with no alteration to Gene A's DNA sequence.

MicroRNAs also offer the possibility of novel therapeutic interventions for the treatment of endometriosis. MicroRNA therapy has already been proposed as a possible new method of the treatment of a range of diseases such as cancer and viral illnesses [448] but at present, this therapy is in the early developmental stages. The basic premise of miRNA therapy is that, if the miRNAs essential for the progression or initiation of a particular disease are known, then it may be possible to create complimentary anti-miRNA strands that would bind to the target miRNA thus, decreasing its ability to regulate gene expression. This would be of particular interest for regressing endometriosis. For example, if it were possible to target anti-miRNA strands to endometriotic cells, rendering their ability to express target genes such as the estrogen receptor, aromatase or anti apoptotic genes, these cells would be stimulated to undergo atrophy. However, this approach should be met with caution since a single miRNA may regulate many genes, thus the possibility of miRNA therapy disrupting non-target genes with unwanted effects must be considered.

3.5.3 Can Epigenetic Aberrations explain the Malignant Potential of Endometriosis?

Deepening of our understanding of the role of epigenetic mechanisms in human cancers has uncovered novel roles for miRNAs in the involvement of cancer origin and progression. In particular the ability of certain miRNAs to regulate genes involved in cellular proliferation, apoptosis and transcriptional regulation has led to them being considered equivalent to oncogenes and tumour suppressors [396, 449]. Recent studies of miRNA expression patterns of endometriosis allows comparative analysis with cancer miRNA patterns (Table 8).

Certain similarities can be observed between the miRNA expression of endometriosis and cancer. For example, several tumour suppressor miRNAs are down regulated in malignant cells and endometriotic cells. Conversely, up regulation of oncogenic miRNAs associated with malignancies does not appear to match the miRNA expression in endometriosis. It may be that a change in oncogenic miRNA expression contributes to malignant transformation of endometriosis. Comparing

cancer in general to endometriosis is however erroneous, as only certain cancer types are associated with endometriosis, most notably ovarian cancer. Both endometrioid and clear cell carcinoma of the ovary have been identified as arising from endometriosis [365, 450-452]. MiRNA-200a and miRNA-200b are reportedly down regulated in endometrioid and clear cell ovarian cancer [397, 453, 454] as well as endometriosis [358], offering a novel perspective on miRNA-200a and 200b as potential mediators of malignant transformation of ovarian endometriosis. Analysis of the predicted targets of miRNA-200a and 200b give clear indications to their role in carcinogenesis from endometriosis (Table 9). Importantly their targets ZEB1 and ZEB2 are involved to the epithelial-mesenchymal transition (EMT) [455, 456], a process that is characterised by the loss of adhesion molecule expression and increased metastasis. It is important to note that several of the adhesion molecules that are under expressed as a result of EMT in cancer are also under expressed in patients with endometriosis [457, 458]. Not only is this an important factor for the metastatic spread of cancerous cells but it may also explain observations of endometriotic cells appearing at distant sites around the body, possibly by lymphatic transport [459, 460]. Therefore, progressive down regulation of miRNA200a and 200b may induce the transformation of ovarian endometriosis to cancerous tissue via the activation of their target genes which induce the already unstable endometriotic cells into a state of malignancy.

In conclusion, epigenetic aberrations reported in endometriosis may provide novel explanations for the mechanisms by which endometriosis can undergo malignant transformation. Aberrant DNA methylation and altered miRNA profiles may 'prime' endometriotic cells for transformation by imbuing these cells with increased proliferation and resistance to apoptosis. However, far more studies are needed to assess the epimutational steps that separate endometriosis and cancer. It may be that a certain sub-type of ovarian cancer exists which can only originate from ovarian endometriosis. However, more detailed histological and molecular analysis of ovarian cancer found in close proximity to ovarian endometriosis would be required.

4. Concluding Remarks and Future Directions

Throughout this thesis various novels concepts have been addressed with the aim of gaining a better understanding of the mechanisms underlying the development and progression of endometriosis. Predicting the prevalence of endometriosis using known risk factors is, in itself, a novel concept that yielded some interesting results. To date the true prevalence of endometriosis can only be estimated for a handful of countries. Presently there is no non-invasive diagnostic test for endometriosis making it difficult to diagnose even in developed countries, therefore prevalence rates for under developed countries are likely to be inaccurate or simply non-existent. A prediction model, such as the one presented in this thesis is the first step towards understanding the global prevalence of the disease and the concurrence of the results obtained from the model with real world data solidify its accuracy. Further development of this model, using more sophisticated epidemiological methods and accurately weighting risk factors will allow for increased accuracy of the model with a further aim of allowing it to predict incident rates as a number per 1000 of the population. This will allow comparisons with data that exist in other countries. Recently the World Endometriosis Research Foundation announced it will be conducting a prospective study to assess the incidence of endometriosis in 10 countries. This data will allow comparisons with the prediction model when it is completed in 2011. If it is found that the results generated by the model do indeed fit with real world data the most interesting results will come from countries that do not fit the model. Countries whose incidence rates do not fall within predicted parameters may offer novel insights into the epidemiology of endometriosis. An example analogous to this would be the French paradox, which states that the French population has a much lower incidence of heart disease than would be expected of a country with a diet so high in saturated fats. Although a satisfactory answer for this paradox has yet to be identified, some claim higher than average consumption of red wines may illicit a protective effect due to their polyphenol content.

Although very few studies have been conducted focussing on the involvement of epigenetics in the pathophysiology of endometriosis, it is clear that modified epigenetic states play a role in its pathogenesis. The over-expression of the DNMTs and aberrant expression of miRNAs appear to be the main contenders. These findings are in agreement with our current understanding of the disease and provide new insights into the aetiology of endometriosis. Examples of epigenetic involvement in endometriosis which have been illustrated include; the establishment of the aromatase positive feedback loop, the contribution of HOXA10 aberrations leading to the observed decreased fertility in endometriosis patients, and the implications that decreased miRNA expression may lead to immune system anomalies (Figure 31). The involvement of aberrant epigenetic mechanisms may also enhance our understanding of the key hereditary features of endometriosis whilst highlighting its manifestation in the absence of any family history. If epigenetic (and genetic) changes predisposing endometriosis occur *in-utero* then endometriosis may be acquired through environmental factors, chemical exposure and dietary components in much the same way as in the initiation and progression of cancer. However, once these epigenetic changes are established they may then be passed onto future generations, increasing susceptibility to endometriosis.

Epigenetics may also provide an insight into the way in which retrograde menstruation and coelomic metaplasia operate synergistically. For example, should the epigenetic changes occur *in-utero*, then they may prime the cells of developing mullerian duct or indeed, any other structure to possess what could be considered 'pre-endometriotic' cells (i.e. cells that, given the correct stimulus, may fully transform to become into endometriotic cells). The stimulus required for transformation of pre-endometriotic cells into endometriotic cells may arise from refluxed menstrual effluence such as growth factors, cytokines and hormones.

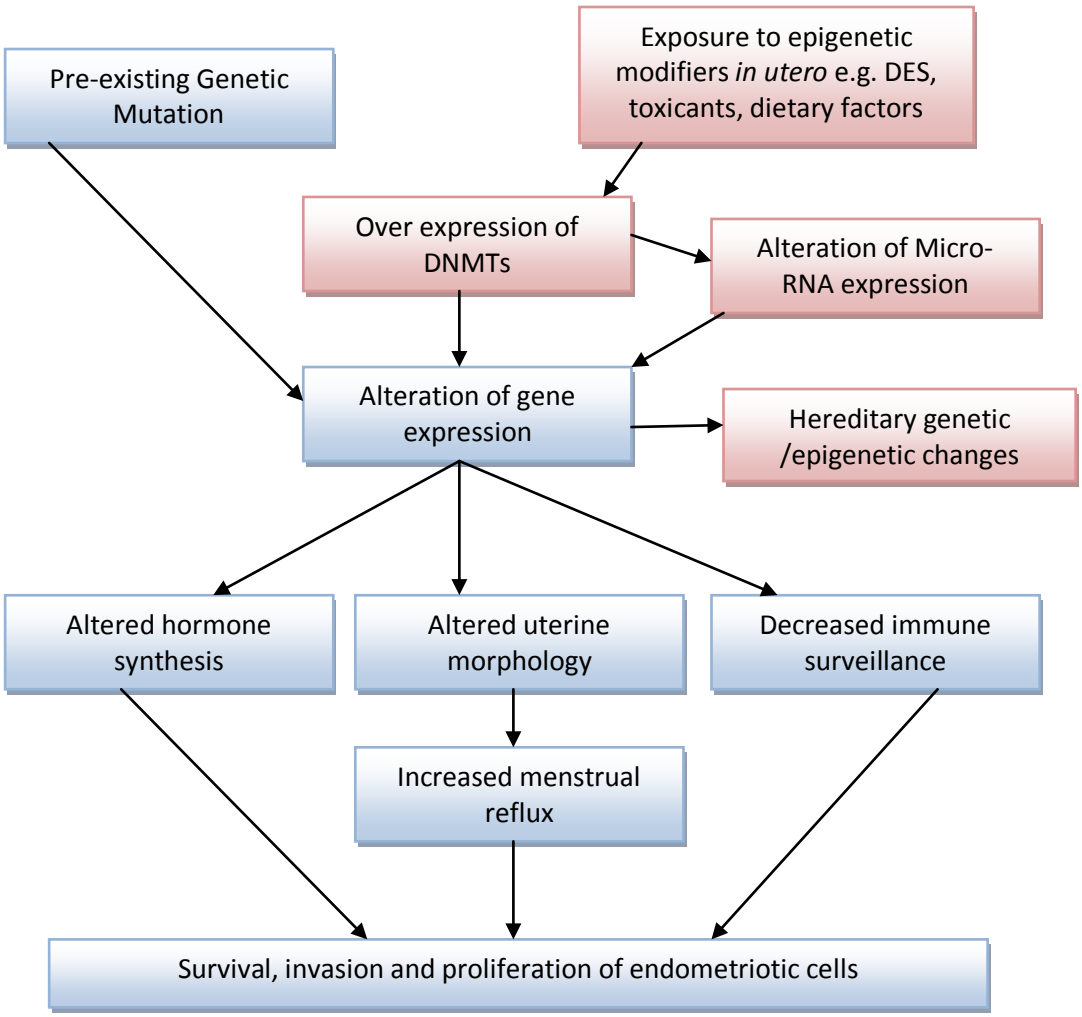


Figure 31. The interactions contributing to the pathogenesis of endometriosis
Factors in blue represent established contributors to the aetiology of endometriosis, factors in red are the novel concepts that have been explored in this thesis

Alterations in the functional development of the uterus as a result of epigenetic deregulation of the HOXA10 gene may predispose to higher volumes of menstrual reflux. Additionally, alteration of the epigenetic regulation of the immune system through the alteration of miRNA action may provide a permissive environment for endometriotic cell survival and proliferation. However, transformation may also result from developmental exposure to certain drugs or compounds that increase levels of mitogenic hormones, such as dioxin or DES. This would explain how non-menstruating women and men can develop an endometriosis like phenotype.

Epigenetics could also provide an interesting new approach for the treatment of endometriosis. Current therapeutic approaches are based on manipulation of the hormonal system, with varied success in relieving pain and restoring fertility. Therefore, utilising drugs that can correct epigenetic errors would be advantageous, whether alone or in combination with existing treatments for endometriosis. These drugs offer a promising new path to follow and have already been cited as a new addition to the arsenal of anti-cancer drugs. New epigenetic therapies for endometriosis could include alterations to diet and lifestyle since both play a major role in the establishment and maintenance of epigenetic states. The potential for dietary therapy for endometriosis based on restoring epigenetic marks may be an option worth consideration.

Dysmenorrhoea is one of the most common symptoms of endometriosis. This intense pain commonly experienced by women is reportedly reduced by supplementation of the diet with fish oils [70], an effect which is enhanced by the addition of vitamin B_{12} [70]. Vitamin B_{12} is a major donor in the production of the methyl donor S-adenosyl methionine (SAM) and is therefore also important for maintaining methylation patterns. Whether or not the relief of dysmenorrhoea by a combination of fish oils and vitamin B_{12} is due to re-establishment of epigenetic marks (such as restoring the methylation state of hypomethylated genes) remains to be clarified. A possible mechanism for the manifestation of dysmenorrhoea is the over production of prostaglandins [461], which may be stimulated by epigenetic hypomethylation in endometriosis as illustrated in the aromatase cycle (Figure 20). It could be postulated that supplementing the diet with vitamin B_{12} or other methyl donors may partially, or completely, restore the methylation patterns of the aromatase and/or SF-1 gene and thus, reduce production of prostaglandins, hence alleviating dysmenorrhoea. Potential dietary benefits that may result in the suppression of endometriosis and its symptoms may also come from phytologically derived isoflavones such as genistein. Supplementation of the diet with genistein has been shown to regress endometriosis in rat models. This regression is thought to occur

via the effect genistein has on epigenetic states. For example, genistein has been shown reactivate epigenetically silenced tumour suppressor genes such as p53 (which is down regulated in endometriosis) in prostate cancer cells [462] via the induction of histone acetylation and demethylation. If p53 function can be restored in endometriotic cells via the same mechanism, this may decrease the proliferative nature of endometriotic cells.

There is no doubt that there are much more exciting discoveries to be made regarding the involvement of epigenetics in endometriosis. Further lines of investigation will endeavour to answer the following questions:

- What are the methylation profiles of the CpG islands and histones of ectopic and eutopic endometrial cells from women with endometriosis?
- What are the consequences of exposure to certain environmental factors on the epigenetic marks in women with endometriosis?
- What effect does exposure to dioxin and other environment factors have on the progeny of those exposed?
- Could demethylating agents such as 5-aza-2'-deoxycytidine be used for the treatment of endometriosis?
- Can epigenetic alterations precede malignant transformation of endometriotic cells?
- Is loss of imprinting a contributory factor to the pathophysiology of endometriosis?
- Can epigenetic biomarkers (such as methylation patterns or miRNA signatures) be used for diagnosis or identifying those at risk of developing endometriosis?

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Appendix 1

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Afghanistan	7.4	-	24.3	-	-
Albania	2.2	-	45.0	17	-
Algeria	2.5	9.08	60.0	3	3.7
Andorra	1.3	-	91.3	-	-
Angola	6.7	11.43	37.2	26	4.5
Argentina	2.3	2.19	90.6	66	8.0
Armenia	1.3	4.92	64.1	4	8.9
Australia	1.7	2.07	92.7	112	8.9
Austria	1.4	2.07	65.8	154	12.9
Azerbaijan	1.8	5.98	49.9	24	8.7
Bangladesh	3.2	31.37	25.0	0	3.4
Belarus	1.2	2.72	71.6	33	11.5
Belgium	1.7	2.18	97.3	131	12.0
Belize	3.1	3.68	48.6	38	3.1
Benin	5.7	-	46.1	14	-
Bhutan	4.2	-	9.1	-	-
Bolivia	3.8	5.45	64.4	25	8.1
Bosnia and Herzegovina	1.3	-	45.3	54	-
Botswana	3.1	5.17	52.5	36	4.7
Brazil	2.3	3.69	84.2	45	7.0
Brunei Darussalam	2.4	-	77.6	1	-
Bulgaria	1.2	3.19	70.5	53	10.0
Burkina Faso	6.6	21.62	18.6	60	2.2

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Burundi	6.8	-	10.6	72	-
Cambodia	4.0	9.33	19.7	3	3.4
Cameroon	4.5	15.44	52.9	40	5.7
Canada	1.5	2.74	81.1	91	11.6
Central African Republic	4.9	-	43.8	17	-
Chad	6.7	-	25.8	3	-
Chile	2.0	3.44	87.7	44	7.8
China	1.7	3.95	40.5	27	3.2
Colombia	2.6	5.17	77.4	18	10.1
Comoros	4.7	-	36.3	1	-
Congo	6.3	16.56	54.4	19	2.1
Costa Rica	2.2	4.13	61.7	20	6.0
Côte d'Ivoire	4.9	24.64	45.8	17	4.9
Croatia	1.3	3.78	59.9	128	14.4
Cuba	1.6	7.70	76.0	27	4.7
Cyprus	1.6	-	69.5	69	7.5
Czech Republic	1.2	2.86	74.5	176	14.3
Democratic People's Republic of Korea	2.0	-	61.7	8	-
Democratic Republic of the Congo	6.7	45.57	32.7	20	2.1
Denmark	1.8	1.53	85.5	131	14.2
Djibouti	4.9	-	84.6	4	-
Dominican Republic	2.7	5.86	60.1	45	3.3
Ecuador	2.7	4.74	62.8	15	6.1
Egypt	3.2	12.51	42.3	1	1.0
El Salvador	2.8	6.76	60.1	20	4.5
Equatorial Guinea	5.9	-	50.0	-	-
Eritrea	5.4	-	20.8	5	-
Estonia	1.4	2.76	69.6	97	14.2
Ethiopia	5.7	19.15	16.2	9	7.3

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Finland	1.7	1.70	60.9	105	10.3
France	1.9	1.68	76.7	93	9.7
Gabon	3.9	5.95	85.2	80	6.6
Gambia	4.6	17.18	26.1	31	1.8
Georgia	1.4	4.85	51.5	11	6.6
Germany	1.3	2.33	88.5	139	12.0
Ghana	4.2	-	46.3	21	-
Greece	1.2	3.49	61.4	64	8.7
Guatemala	4.5	-	47.2	7	-
Guinea	5.8	26.60	36.5	2	4.3
Guinea-Bissau	7.1	-	35.6	18	-
Guyana	2.2	5.66	38.5	21	6.5
Haiti	3.9	12.72	38.8	11	1.9
Honduras	3.6	-	46.4	17	-
Hungary	1.3	2.21	65.9	114	11.1
Iceland	2.0	1.45	93.0	64	17.0
India	3.0	11.25	28.7	1	4.9
Indonesia	2.3	21.65	47.9	0	8.1
Iran (Islamic Republic of)	2.1	9.32	68.1	0	2.3
Iraq	4.7	-	66.8	-	-
Ireland	1.9	2.10	60.4	211	13.8
Israel	2.8	3.97	91.7	16	8.6
Italy	1.3	2.87	67.5	79	9.3
Jamaica	2.4	-	52.2	22	4.7
Japan	1.3	3.38	65.7	49	6.4
Jordan	3.4	7.93	79.3	2	3.5
Kazakhstan	1.9	2.77	55.9	24	10.0
Kenya	5.0	7.15	41.6	11	6.5
Kuwait	2.3	3.66	96.4	0	6.2

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Kyrgyzstan	2.6	4.01	33.7	10	10.1
Lao People's Democratic Republic	4.7	-	21.6	17	-
Latvia	1.3	2.73	65.9	65	14.0
Lebanon	2.3	5.26	88.0	10	5.4
Lesotho	3.5	21.91	18.2	29	3.2
Liberia	6.8	-	47.9	6	-
Libyan Arab Jamahiriya	2.9	-	86.9	0	-
Lithuania	1.3	3.19	66.6	95	16.6
Luxembourg	1.7	-	92.4	-	12.7
Madagascar	5.3	-	27.0	8	-
Malawi	6.0	37.30	17.2	15	1.8
Malaysia	2.8	4.52	65.1	5	8.4
Maldives	4.1	-	29.7	8	-
Mali	6.8	10.10	33.7	5	2.0
Malta	1.5	-	92.1	44	10.9
Mauritania	5.7	-	64.3	0	-
Mexico	2.3	4.25	76.0	49	7.4
Monaco	1.8	-	100.0	-	-
Mongolia	2.4	1.69	57.0	12	3.7
Morocco	2.7	13.22	58.8	3	3.4
Mozambique	5.4	42.84	38.0	4	2.9
Myanmar	2.3	20.25	30.6	0	4.8
Namibia	3.8	4.86	33.5	11	3.5
Nepal	3.6	-	15.8	0	-
Netherlands	1.7	1.90	66.8	102	12.2
New Zealand	2.0	2.09	86.0	94	12.4
Nicaragua	3.2	8.48	58.1	14	6.9
Niger	7.8	16.09	23.3	0	6.4
Nigeria	5.7	30.36	48.3	72	3.2

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Norway	1.8	1.95	80.5	72	13.9
Oman	3.6	-	78.6	-	5.3
Pakistan	4.1	4.47	34.8	0	9.8
Panama	2.7	3.20	57.8	49	6.6
Papua New Guinea	3.9	-	13.2	-	5.7
Paraguay	3.8	4.25	58.5	50	7.5
Peru	2.8	6.78	74.6	29	8.7
Philippines	3.1	5.94	62.6	15	11.6
Poland	1.2	2.89	62.0	88	12.5
Portugal	1.5	2.43	55.6	124	8.6
Qatar	2.9	-	92.3	-	6.5
Republic of Korea	1.2	5.79	80.8	69	5.7
Republic of Moldova	1.2	5.35	46.3	42	7.4
Romania	1.3	3.79	54.7	85	9.4
Russian Federation	1.3	3.54	73.3	69	9.1
Rwanda	5.6	30.01	21.8	49	8.8
Saint Lucia	2.2	-	31.3	61	-
Saudi Arabia	3.9	5.92	88.5	0	4.5
Senegal	4.9	9.81	51.0	4	4.4
Serbia and Montenegro	1.6	-	52.3	75	-
Sierra Leone	6.5	-	40.2	44	-
Singapore	1.3	-	100.0	-	11.1
Slovakia	1.2	3.14	58.0	103	12.4
Slovenia	1.2	2.08	50.8	82	10.6
Solomon Islands	4.2	-	17.1	1	-
Somalia	6.3	-	35.9	-	-
South Africa	2.8	6.94	57.9	87	5.4
Spain	1.3	2.60	76.7	106	9.9
Sri Lanka	1.9	14.68	21.0	1	9.0

Country	Fertility – Avg children per woman. Source WHO (2004)	Meat vs Plant Ratio – Consumption in g/capita/day. Source FAO (2001-2003)	% Population In Urban Areas Source WHO (2005)	Alcohol consumption – Kg/capita/yr. Source FAO(2003)	Ovarian Cancer – Incidence rates per 100,000. Source Globocan (2002)
Sudan	4.3	3.94	40.8	0	4.3
Suriname	2.6	6.75	77.2	45	6.0
Swaziland	3.8	6.95	23.9	64	3.1
Sweden	1.7	1.94	83.4	77	11.4
Switzerland	1.4	1.92	67.5	102	12.4
Syrian Arab Republic	3.3	-	50.3	0	-
Tajikistan	3.7	9.84	24.2	1	7.0
Thailand	1.9	6.75	32.5	35	5.6
The former Yugoslav Republic of Macedonia	1.5	4.43	59.7	50	9.2
Togo	5.2	-	36.3	14	-
Tunisia	1.9	8.31	64.4	1	2.7
Turkey	2.4	8.79	67.3	11	4.8
Turkmenistan	2.7	4.19	45.8	7	9.3
Uganda	7.1	16.18	12.4	133	5.9
Ukraine	1.1	4.23	67.3	44	10.9
United Arab Emirates	2.5	-	85.5	-	4.1
United Kingdom	1.7	2.36	89.2	124	13.4
United Republic of Tanzania	4.9	14.53	37.5	67	4.0
United States of America	2.0	2.67	80.8	98	10.6
Uruguay	2.3	2.21	93.0	48	8.7
Uzbekistan	2.7	4.77	36.4	6	8.2
Venezuela (Bolivarian Republic of)	2.7	5.21	88.1	77	5.9
Viet Nam	2.3	7.60	26.7	9	4.2
Yemen	6.0	13.04	26.3	0	2.8
Zambia	5.5	18.86	36.5	27	4.1
Zimbabwe	3.4	11.40	35.9	25	7.2

Appendix 2

Country	Mean age at menarche (years)	Age at menarche Year	HDI Score	HDI Year
Algeria	14.30	1993	0.672	1995
Argentina	12.59	1995	0.836	1995
Australia	13.00	1998	0.949	2000
Bangladesh	15.80	1993	0.453	1995
Belgium	13.00	1984	0.883	1985
Britain	13.30	1986	0.87	1985
Cameroon	14.61	1985	0.523	1985
Chile	13.00	1998	0.845	2000
China	12.38	1997	0.691	2000
Columbia	12.80	1991	0.729	1990
Congo-Brazza	12.00	1992	0.559	1990
Congo-Kinshasa	13.83	1984	0.43	1985
Denmark	13.00	1998	0.935	2000
Dominican republic	12.60	1990	0.697	1990
Egypt	13.20	1978	0.482	1980
Finland	13.20	1984	0.884	1985
France	13.05	1979	0.872	1980
Germany (east)	14.00	1998	0.928	2000
Ghana	13.98	1989	0.517	1990
Greece	12.00	1984	0.869	1985
Guatemala	13.75	1995	0.626	1995
Haiti	15.37	1995	0.487	1995
Hungary	12.90	1993	0.817	1995
Iceland	13.06	1978	0.89	1980
India (Punjab)	14.31	1980	0.45	1980
Indonesia	13.00	1990	0.626	1990
Ireland	13.52	1986	0.851	1985
Israel	13.29	1982	0.83	1980
Italy	12.20	1992	0.892	1990
Jamaica	13.10	1988	0.713	1990
Japan	12.50	1986	0.899	1985
Kenya	14.40	1987	0.534	1985
Malaysia	14.20	1985	0.696	1985
Mexico	12.40	1993	0.786	1995
Morocco	13.75	1996	0.581	1995
Mozambique	13.20	2000	0.375	2000
Nepal (high altitude)	16.20	1983	0.38	1985
New Zealand	12.90	1994	0.908	1995
Nicaragua	14.00	1971	0.583	1975
Nigeria	15.00	1998	0.445	2000
Norway	13.20	1995	0.938	1995
Papua New Guinea	15.80	1992	0.495	1990

Country	Mean age at menarche (years)	Age at menarche Year	HDI Score	HDI Year
Peru	13.23	1988	0.71	1990
Philippines	13.60	1988	0.721	1990
Poland	13.06	1982	0.806	1990
Portugal	12.03	2003	0.897	2005
Romania	13.47	1967	0.786	1980
Russia	13.00	1997	0.782	2000
Senegal	16.10	1997	0.473	2000
South Korea	13.90	1986	0.785	1985
Spain	12.31	1997	0.932	2000
Sri Lanka	13.50	1983	0.683	1985
Sudan	13.75	1983	0.4	1985
Sweden	13.09	1976	0.872	1975
Switzerland	13.00	1996	0.926	1995
Tanzania	15.21	1971	0.421	1990
Thailand	12.30	1997	0.761	2000
Turkey	13.28	1996	0.717	1995
United States	12.80	1991	0.919	1990
Venezuela	12.68	1981	0.737	1980
Yemen	14.40	1979	0.402	1990
Zambia	13.70	1980	0.478	1980
Zimbabwe	13.50	1995	0.613	1995

Appendix 3

	Rank Score						
Country	**Fertility**	**Plant vs Meat consumption Ratio**	**% in Urban Environment**	**Alcohol Consumption**	**Ovarian Cancer**	**Total score**	**ERI**
Algeria	4	3	3	1	2	13	0.52
Angola	2	2	2	2	2	10	0.4
Argentina	4	5	5	4	3	21	0.84
Armenia	4	4	4	1	3	16	0.64
Australia	4	5	5	5	3	22	0.88
Austria	4	5	4	5	5	23	0.92
Azerbaijan	4	4	3	2	3	16	0.64
Bangladesh	3	1	2	1	2	9	0.36
Belarus	4	5	4	2	4	19	0.76
Belgium	4	5	5	5	5	24	0.96
Belize	3	4	3	2	2	14	0.56
Bolivia	3	4	4	2	3	16	0.64
Botswana	3	4	3	2	2	14	0.56
Brazil	4	4	5	3	3	19	0.76
Bulgaria	4	4	4	3	4	19	0.76
Burkina Faso	2	1	1	3	1	8	0.32
Cambodia	3	3	1	1	2	10	0.4
Cameroon	3	1	3	2	2	11	0.44
Canada	4	5	5	5	4	23	0.92
Chile	4	4	5	3	3	19	0.76
China	4	4	3	2	2	15	0.6
Colombia	4	4	4	1	4	17	0.68
Congo	2	1	3	1	1	8	0.32
Costa Rica	4	4	4	2	3	17	0.68

Country	Fertility	Plant vs Meat consumption Ratio	% in Urban Environment	Alcohol Consumption	Ovarian Cancer	Total score	ERI
Côte d'Ivoire	3	1	3	1	2	10	0.4
Croatia	4	4	3	5	5	21	0.84
Cuba	4	3	4	2	2	15	0.6
Czech Republic	4	5	4	5	5	23	0.92
Democratic Republic of the Congo	2	1	2	1	1	7	0.28
Denmark	4	5	5	5	5	24	0.96
Dominican Republic	4	4	4	3	2	17	0.68
Ecuador	4	4	4	1	3	16	0.64
Egypt	3	2	3	1	1	10	0.4
El Salvador	4	3	4	1	2	14	0.56
Estonia	4	5	4	5	5	23	0.92
Ethiopia	2	1	1	1	3	8	0.32
Finland	4	5	4	5	4	22	0.88
France	4	5	4	5	4	22	0.88
Gabon	3	4	5	4	3	19	0.76
Gambia	3	1	2	2	1	9	0.36
Georgia	4	4	3	1	3	15	0.6
Germany	4	5	5	5	5	24	0.96
Greece	4	4	4	4	3	19	0.76
Guinea	2	1	2	1	2	8	0.32
Guyana	4	4	2	2	3	15	0.6
Haiti	3	2	2	1	1	9	0.36
Hungary	4	5	4	5	4	22	0.88
Iceland	4	5	5	4	5	23	0.92
India	3	2	3	1	2	11	0.44
Indonesia	4	1	3	1	3	12	0.48
Iran (Islamic Republic of)	4	3	4	1	1	13	0.52
Ireland	4	5	4	5	5	23	0.92
Israel	4	4	5	1	3	17	0.68
Italy	4	5	4	4	4	21	0.84
Japan	4	4	4	3	3	18	0.72

Country	Fertility	Plant vs Meat consumption Ratio	% in Urban Environment	Alcohol Consumption	Ovarian Cancer	Total score	ERI
Jordan	3	3	4	1	2	13	0.52
Kazakhstan	4	5	3	2	4	18	0.72
Kenya	2	3	3	1	3	12	0.48
Kuwait	4	4	5	1	3	17	0.68
Kyrgyzstan	4	4	2	1	4	15	0.6
Latvia	4	5	4	4	5	22	0.88
Lebanon	4	4	5	1	2	16	0.64
Lesotho	3	1	1	2	2	9	0.36
Lithuania	4	4	4	5	5	22	0.88
Malawi	2	1	1	1	1	6	0.24
Malaysia	4	4	4	1	3	16	0.64
Mali	2	2	2	1	1	8	0.32
Mexico	4	4	4	3	3	18	0.72
Mongolia	4	5	3	1	2	15	0.6
Morocco	4	1	3	1	2	11	0.44
Mozambique	2	1	2	1	1	7	0.28
Myanmar	4	1	2	1	2	10	0.4
Namibia	3	4	2	1	2	12	0.48
Netherlands	4	5	4	5	5	23	0.92
New Zealand	4	5	5	5	5	24	0.96
Nicaragua	3	3	3	1	3	13	0.52
Niger	1	1	2	1	3	8	0.32
Nigeria	2	1	3	4	2	12	0.48
Norway	4	5	5	4	4	22	0.88
Pakistan	3	4	2	1	3	13	0.52
Panama	4	4	3	3	3	17	0.68
Paraguay	3	4	3	3	3	16	0.64
Peru	4	3	4	2	3	16	0.64
Philippines	3	4	4	1	4	16	0.64
Poland	4	5	4	5	5	23	0.92
Portugal	4	5	3	5	3	20	0.8

Country	Fertility	Plant vs Meat consumption Ratio	% in Urban Environment	Alcohol Consumption	Ovarian Cancer	Total score	ERI
Republic of Korea	4	4	5	4	2	19	0.76
Republic of Moldova	4	4	3	3	3	17	0.68
Romania	4	4	3	5	4	20	0.8
Russian Federation	4	4	4	4	4	20	0.8
Rwanda	2	1	2	3	3	11	0.44
Saudi Arabia	3	4	5	1	2	15	0.6
Senegal	3	3	3	1	2	12	0.48
Slovakia	4	4	3	5	5	21	0.84
Slovenia	4	5	3	5	4	21	0.84
South Africa	4	3	3	5	2	17	0.68
Spain	4	5	4	5	4	22	0.88
Sri Lanka	4	1	2	1	4	12	0.48
Sudan	3	4	3	1	2	13	0.52
Suriname	4	3	4	3	3	17	0.68
Swaziland	3	3	2	4	2	14	0.56
Sweden	4	5	5	4	4	22	0.88
Switzerland	4	5	4	5	5	23	0.92
Tajikistan	3	3	2	1	3	12	0.48
Thailand	4	3	2	2	2	13	0.52
The former Yugoslav Republic of Macedonia	4	4	3	3	4	18	0.72
Tunisia	4	3	4	1	1	13	0.52
Turkey	4	3	4	1	2	14	0.56
Turkmenistan	4	4	3	1	4	16	0.64
Uganda	1	1	1	5	2	10	0.4
Ukraine	4	4	4	3	4	19	0.76
United Kingdom	4	5	5	5	5	24	0.96
United Republic of Tanzania	3	1	2	4	2	12	0.48
United States of America	4	5	5	5	4	23	0.92
Uruguay	4	5	5	3	3	20	0.8
Uzbekistan	4	4	2	1	3	14	0.56

Country	Fertility	Plant vs Meat consumption Ratio	% in Urban Environment	Alcohol Consumption	Ovarian Cancer	Total score	ERI
Venezuela (Bolivarian Republic of)	4	4	5	4	2	19	0.76
Viet Nam	4	4	2	1	2	13	0.52
Yemen	2	1	2	1	1	7	0.28
Zambia	2	1	2	2	2	9	0.36
Zimbabwe	3	2	2	2	3	12	0.48