Genetic Variation and Risk of Endometrial Cancer

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DECLARATION

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Date **KATIE A. ASHTON**

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TABLE OF CONTENTS

Declaration i

Acknowledgements ii

Table of Contents iii

Abstract vi

Chapter I **Introduction** 1

1.1 **Endometrial Cancer** 2

1.2 **Incidence and Death Rates of Endometrial Cancer** 3

1.3 **Symptoms, Diagnosis and Treatment of Endometrial Cancer** 5

1.4 **The Role of Hormones in Endometrial Cancer** 6

1.4.1 Estrogens 7

1.4.2 Progesterone 8

1.5 **Risk Factors Associated with the Development of Endometrial Cancer** 8

1.6 **Estrogen Dependent and Independent Endometrial Cancer** 10

1.6.1 Genetic Alterations in Estrogen Dependent Endometrial Carcinomas 11

1.6.1.1 *Microsatellite Instability (MSI)* 11

1.6.1.2 *K-ras* 11

1.6.1.3 *Beta-catenin* 12

1.6.1.4 *PTEN* 12

1.6.2 Genetic Alterations in Estrogen Independent Endometrial Carcinomas 13

1.6.2.1 *HER2/neu* 13

1.6.2.2 *TP53* 13

1.7 **Genetic Alterations in Hereditary and Sporadic Endometrial Cancer** 14

1.8 **Types of DNA Sequence Variations** 15

1.9 **The Relationship between Hormone Biosynthesis and Endometrial Cancer** 17

1.9.1 CYP11A1 18

1.9.2 CYP17A1 19

1.9.3 CYP19A1 19

1.9.4 HSD17β1 and HSD17β2 20

1.10 The Relationship between Hormone Receptors and Endometrial Cancer 20
1.10.1 Estrogen Receptor α and β (ESR1 and ESR2) 20
1.10.2 Progesterone Receptor (PGR) 21
1.10.3 Androgen Receptor (AR) 22
1.10.4 Amplified in Breast Cancer 1 (AIB1) 23
1.11 The Relationship between Estrogen Metabolism and Endometrial Cancer 24
1.11.1 Phase I Estrogen Metabolism 25
1.11.1.1 CYP1A1 26
1.11.1.2 CYP1A2 26
1.11.1.3 CYP1B1 27
1.11.2 Phase II Estrogen Metabolism 28
1.11.2.1 COMT 28
1.11.2.2 Glutathione S Transferases (GSTs) 28
1.11.2.3 SULT1E1 29
1.11.2.4 UGT1A1 30
1.12 The Relationship between Testosterone Metabolism and Endometrial Cancer 30
1.12.1 5α-reductase II (SRD5A2) 31
1.13 The Relationship between Genes involved in DNA Repair and Cell Cycle Control, and Endometrial Cancer 31
1.13.1 Cyclin D1 32
1.13.2 MUTYH 32
1.13.3 TP53 and MDM2 34
1.14 Summary 36

Aims 36

Statement I Author Contribution to Chapter II Manuscript 37

Chapter II The Association of the COMT V158M Polymorphism with Endometrial/Ovarian Cancer in HNPCC families adhering to the Amsterdam Criteria 38

Statement II Author Contribution to Chapters III-VI Manuscripts 48

Chapter III Polymorphisms in genes of the steroid hormone biosynthesis and metabolism pathways and endometrial cancer risk 50

Chapter IV Estrogen receptor polymorphisms and the risk of endometrial cancer 76

Chapter V Polymorphisms in TP53 and MDM2 combined are associated with high grade endometrial cancer 86

Chapter VI The influence of the Cyclin D1 870 G>A polymorphism as an endometrial cancer risk factor 93

Statement III Author Contribution to Chapter VII Manuscript 100

Chapter VII Genetic variants in MUTYH are not associated with endometrial cancer risk 101

Chapter VIII General Discussion 107

8.1 HNPCC Related Endometrial Cancer and Genetic Variation in Estrogen Metabolism 109

8.2 Polymorphisms in Hormone Biosynthesis and Hormone Receptors Genes and Risk of Endometrial Cancer 110

8.2.1 Estrogens Receptors (ESR1 and ESR2) 111

8.2.2 Androgen Receptor (AR) 111

8.3 Polymorphisms in Estrogen Metabolism Genes and Risk of Endometrial Cancer 112

8.4 Polymorphisms in DNA Repair and Cell Cycle Control Genes, and Risk of Endometrial Cancer 113

8.5 Overall Conclusions 114

8.6 Recommendations 115

8.7 Summary 116

References 117

ABSTRACT

Endometrial cancer is one of the most common female cancers in industrialized countries. Traditional risk factors associated with endometrial cancer are well understood and include excessive exposure to estrogen or estrogen unopposed by progesterone. However, variations in the genes that influence these hormones and their association with endometrial cancer have not been well investigated. By studying genetic variation in endometrial cancer, novel markers of risk may be discovered that can be used to identify women at high risk and for the implementation of specialised treatments. Polymorphisms in the genes involved in the following pathways; hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair and cell cycle control, have been suggested to be involved in the initiation and development of endometrial cancer. The focus of this study was to examine genetic variants in these pathways to assess the existence of an association with the risk of endometrial cancer.

In the first part of this study, the COMT V158M polymorphism was examined in a hereditary non-polyposis colorectal cancer (HNPCC) cohort to determine its association with disease expression. The heterozygous genotype was over-represented in women with endometrial/ovarian cancer that did not harbour mismatch repair (MMR) gene mutations. This result suggested that the COMT V158M polymorphism may alter the risk of developing HNPCC related endometrial/ovarian cancer in MMR mutation negative women. Since COMT is involved in the metabolism of estrogen and that estrogen is the main risk factor for endometrial cancer development, closer examination was warranted to determine the association of genetic variation involved in hormone-related pathways and endometrial cancer risk, outside of the context of an inherited predisposition to disease.

In the second part of this study, a cohort of 191 women with endometrial cancer and 291 healthy control women were genotyped for polymorphisms in genes involved in hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair and cell cycle control. The results revealed that variations in estrogen receptor alpha (ESR1) and beta (ESR2), and the androgen receptor (AR), were associated with an increase and decrease in endometrial cancer risk, respectively. Additionally, polymorphisms in CYP1A1, CYP1B1, GSTM1 and GSTP1 were related to a decrease in endometrial cancer risk. A trend was observed for the cyclin D1 870 G>A polymorphism and an increase in endometrial cancer risk, however, this result did not

reach significance. Taken together, these results revealed that perturbations in the hormone receptors and estrogen metabolism genes, may aid in the identification of women at high risk of developing endometrial cancer. Interestingly, stratification of the women with endometrial cancer revealed that combinations of polymorphisms in TP53 and MDM2 were associated with higher grades of cancer. This finding may possibly have significant implications as women with reduced apoptotic ability, due to combinations of polymorphisms in these genes, have an increased risk of presenting with higher grades of endometrial cancer, that are associated with lower survival rates.

In summary, the results of this thesis showed that variation in the estrogen and androgen receptors, and estrogen metabolism genes, may alter the risk of developing endometrial cancer. Moreover, polymorphisms in the cell cycle control genes, TP53 and MDM2, appear to be associated with higher grades of endometrial cancer. This study of polymorphisms may help explain genetic differences in individual susceptibility to endometrial cancer and are markers of risk that aid in the development of effective and personalised strategies to prevent disease development.

This study has improved the understanding of genetic variation associated with endometrial cancer risk. It has the potential to enhance our ability to treat women with endometrial cancer through improved identification and treatment strategies, by virtue of the genetic variation identified, that appears to predispose to disease.

Chapter I

Introduction

Cancer is the leading cause of death in Australia with 1 in 3 men and 1 in 4 women directly affected by the age of 75 (AIHW 2007). The most common cancers in the developed world are colorectal, lung, breast and prostate cancer (AIHW 2007). In the context of oncological disorders of the female genital tract, endometrial cancer is recognised as the most common worldwide (Akhmedkhanov *et al.* 2001; Bray *et al.* 2005). It is the fourth most common cancer in women after lung, colorectal and breast cancer (AIHW 2007).

Endometrial cancer is a serious health issue which has long been a neglected disease. Over the past several decades, the incidence of endometrial cancer has increased and it is well known that the main risk factor for endometrial cancer is excessive exposure to estrogen or estrogen unopposed by progesterone. The environmental and reproductive risk factors associated with altered estrogen and progesterone levels have been elucidated however variations in the genes that influence the effects of estrogen and progesterone have not as yet been extensively studied. The rapid changes in the incidence of endometrial cancer are highly likely to be a result of a complex genetic predisposition to develop endometrial cancer which is influenced by environmental and/or reproductive risk factors. These insults facilitate excessive exposure to exogenous and/or endogenous estrogen or alter the production of progesterone. Little is known about inherited factors that influence endometrial cancer risk and this study focuses on the identification of genetic variation associated with endometrial cancer.

1.1 Endometrial Cancer

The endometrium is the lining of the uterus and is an important female reproductive organ. Alterations in the levels of hormones that occur throughout a woman's menstrual cycle cause the endometrium to change. During the early stages of the menstrual cycle, higher levels of estrogen are produced by the ovaries and the endometrium thickens. After ovulation, estrogen levels drop and progesterone levels increase. If pregnancy does not occur, both estrogen and progesterone levels decrease, leading to menstruation. This cycle continues until a woman reaches menopause. Altered levels of hormones however, can cause abnormal growth of the endometrium and may lead to the development of endometrial cancer (Purdie *et al.* 2001), a tumour of the lining of the uterus, see figure 1. Endometrial cancer often develops over a period of years and is most commonly diagnosed in post menopausal women (Purdie *et al.* 2001).

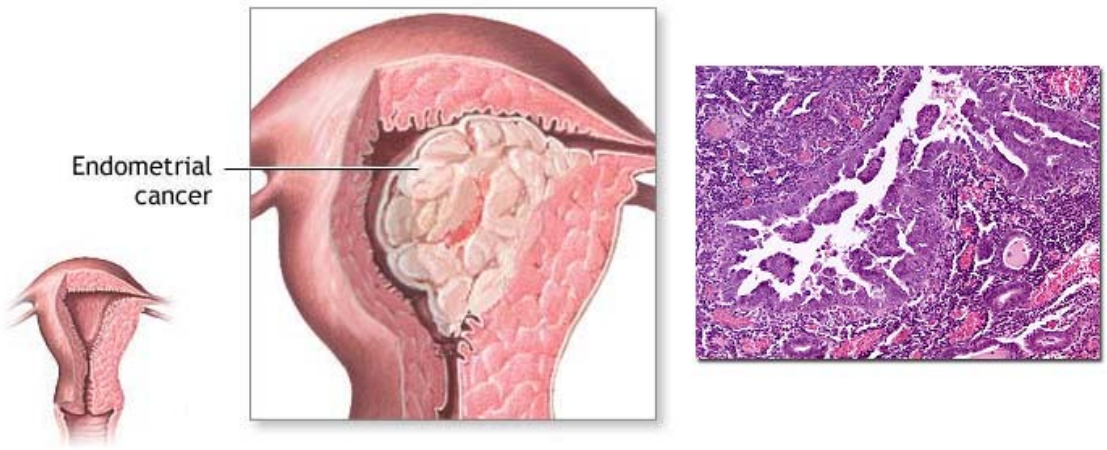

Figure 1: The picture on the left shows a normal uterus with no disease whereas the picture in the middle shows the development of an endometrial carcinoma (www.lifespan.com). The picture on the right shows a microscopic view (10x magnification) of an endometrial adenocarcinoma section with a haematoxylin and eosin (H & E) stain (http://www.microscopyu.com/galleries/pathology/endometriumadenocarcinomalarge1.html).

1.2 Incidence and Death Rates of Endometrial Cancer

The incidence of endometrial cancer has continued to increase from the late 1980's (figures 2 and 3) however, the death rates have decreased significantly, see figure 4 (Kufe *et al.* 2006; AIHW 2007). The annual incidence in Western countries is approximately 10-20 per 100 000 women (Ryan *et al.* 2005) and in Australia, endometrial cancer accounts for approximately 2-4 per 100 000 deaths every year (AIHW 2007). Approximately 1 in 75 women will develop disease by 75 years of age and the average 5 year survival rate is approximately 80% (AIHW 2007). Survival rates differ from 93.7% for localised disease, 67.7% for regional spread and 27.4% for distant metastases (AIHW 2007). After ovarian and cervical cancer, endometrial cancer is the third most common cause of death among women with gynaecological cancers (AIHW 2007). While endometrial cancers are far more common in Caucasians, African-American women developing this disease have a higher death rate as they are diagnosed more commonly with unfavourable types of carcinomas that are difficult to treat and are often far more aggressive (Plaxe *et al.* 1997; Sherman *et al.* 2003). Despite a relative high survival rate of women with endometrial cancer, it remains a significant burden on society.

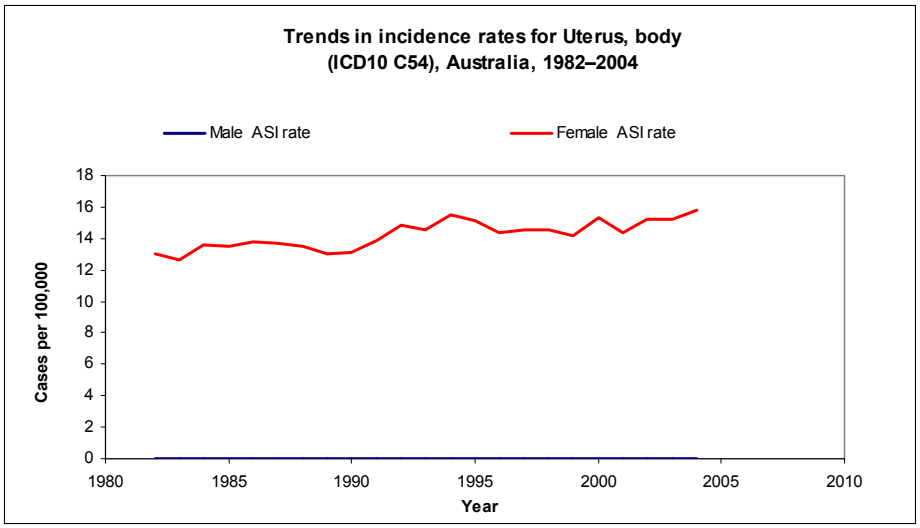

Figure 2: Trends in Incidence Rates for Endometrial Cancer in Australia from 1982 to 2004 (AIHW 2007)

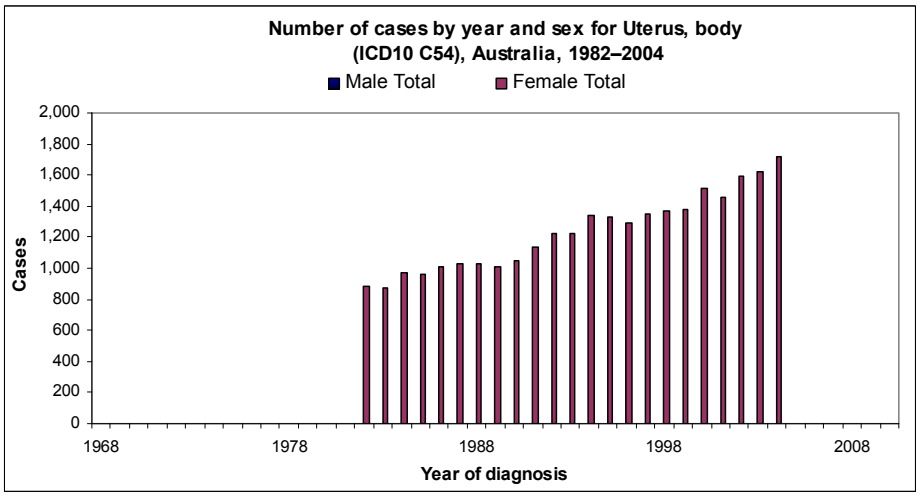

Figure 3: Number of Cases by Year for Endometrial Cancer in Australia from 1982 to 2004 (AIHW 2007)

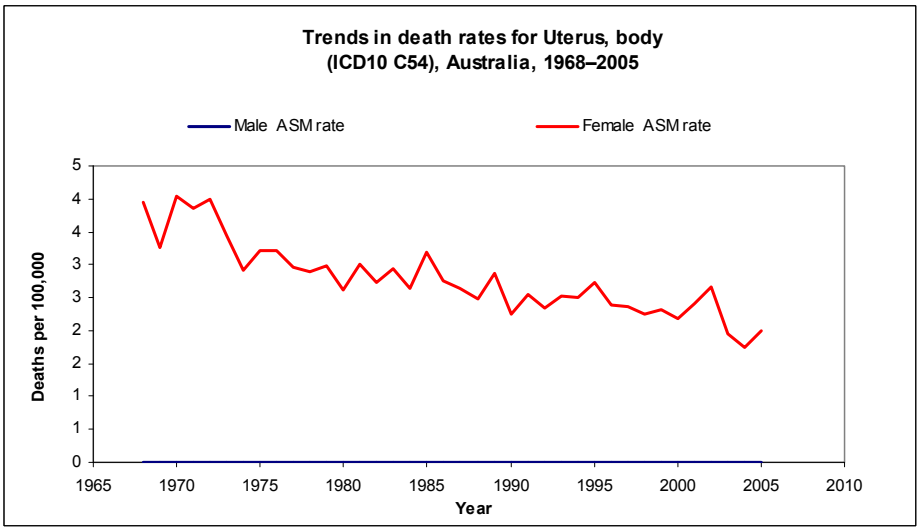

Figure 4: Trends in Death Rates for Endometrial Cancer in Australia from 1968 to 2005 (AIHW 2007)

1.3 Symptoms, Diagnosis and Treatment of Endometrial Cancer

Endometrial cancer usually occurs after menopause and presents firstly as endometrial hyperplasia (Boruban *et al.* 2008). The average age of affected patients is between 50 and 59 years of age (Akhmedkhanov *et al.* 2001). The most common symptom is vaginal bleeding (bleeding between periods for premenopausal women) however a number of other symptoms such as vaginal discharge and pain in the pelvic area can be signs of the disease (Bakkum-Gamez *et al.* 2008). A diagnosis of endometrial cancer frequently involves any combination of the following: a pelvic examination, Pap smear, endometrial curettage, a dilation and curettage (D&C) procedure, biopsy, and/or ultrasound (Bakkum-Gamez *et al.* 2008). If endometrial carcinoma is detected, it is surgically staged and the degree of histological differentiation of carcinoma is assessed using the International Federation of Gynaecology and Obstetrics (FIGO) staging and grading system, described below (Shepherd 1989).

Staging:

- Stage IA: tumour limited to endometrium.
- Stage IB: invasion to less than 50% of the myometrium.
- Stage IC: invasion to greater than 50% of the myometrium.
- Stage IIA: endocervical glandular involvement only.
- Stage IIB: cervical stromal invasion.
- Stage IIIA: tumour invades serosa and/or adnexa and/or positive peritoneal cytology.
- Stage IIIB: vaginal metastases.
- Stage IIIC: metastases of pelvic and/or para-aortic lymph nodes.
- Stage IVA: tumour invasion of bladder and/or bowel mucosa.
- Stage IVB: distant metastases including intra-abdominal and/or inguinal lymph nodes.

Grading:

- Grade 1**:** Well-differentiated cancers have very clear boundaries and cells that look relatively normal. They normally do not grow and spread rapidly.
- Grade 2: Moderately differentiated cancer has more abnormal looking cells and cell boundaries.
- Grade 3**:** Poorly differentiated cancers have less clearly defined boundaries and cells that look very abnormal. They often grow and spread rapidly.

The most common histopathological diagnosis of endometrial carcinomas is an endometriod adenocarcinoma and the primary treatment is surgery, a total hysterectomy (Bakkum-Gamez *et al.* 2008). For patients that have stage I or II disease and a high risk of recurrence, radiotherapy is recommended and for stage III or IV disease, chemotherapy may be considered (Bakkum-Gamez *et al.* 2008). Hormonal therapy including progestins and anti-estrogens is an option and has been found to be particularly useful for stromal sarcomas (Reich *et al.* 2007). In addition, hormonal therapy is a common option for those who wish to preserve their fertility (Bakkum-Gamez *et al.* 2008).

1.4 The Role of Hormones in Endometrial Cancer

Endometrial cancer and other hormone related cancers share a unique mechanism of carcinogenesis (Henderson *et al.* 2000). Cell growth is influenced by endogenous and exogenous hormones (Henderson *et al.* 2000) and for women with endometrial cancer, excessive exposure to estrogen or unopposed estrogen (without progesterone) are the main risk factor for disease development (Bakkum-Gamez *et al.* 2008). The normal function of estrogen and progesterone is to stimulate the growth of the lining of the uterus by triggering proliferation of endometrial cells however during menstruation, the level of these hormones decline and the lining of the uterus is removed. This cycle continues many times throughout a woman's life and a higher number of cycles are associated with a greater number of cell division events (Hecht *et al.* 2006). An increase in the risk of developing endometrial cancer has been proposed to occur through two different mechanisms: 1. Estrogen can stimulate the division of cells that already have mutations and; 2. Excessive cellular proliferation increases the chance of developing new spontaneous mutations (Lord *et al.* 2002).

In pre-menopausal women, the risk of developing endometrial cancer is related to the mitotic activity in the endometrium in the first half of the menstrual cycle (Henderson *et al.* 2000). At this particular point of the cycle, estrogen is unopposed by progesterone (Henderson *et al.* 2000). In post-menopausal women, endometrial cancer risk is mainly associated with increasing body fat, since the amount of adipose tissue is directly related to increasing levels of circulating estrogen (Simpson 2003).

An increase in cellular proliferation due to the hormonal influences of estrogen and progesterone are associated with the accumulation of random genetic errors that can lead to the development of endometrial cancer (Boruban *et al.* 2008). Similar to colorectal cancer, the conversion from normal endometrium to carcinoma has been

proposed to involve variations in genes that support proliferation but inhibit apoptosis and angiogenesis (Enomoto *et al.* 1991). The ability of estrogen and progesterone to induce cellular modifications is an important factor to determine the precise mechanisms that are involved in the initiation and development of endometrial cancer.

1.4.1 Estrogens

Estrogens induce cellular alterations through a number of different systems. Two pathways distinguish the main mechanisms by which estrogen acts; the classical and the non-genomic pathway (Deroo *et al.* 2006). The classical pathway involves estrogen entering the nucleus and binding to the estrogen receptors. The activation of the estrogen receptors allows for estrogen response elements (EREs) to bind to the receptors to initiate protein-protein interactions for the recruitment of coactivators and corepressors to induce a physiological response (Deroo *et al.* 2006). The non-genomic pathway describes how estrogen can act more quickly through interaction with the estrogen receptor when it is located close to the plasma membrane. This pathway results in increased levels of calcium ions (Ca2+) and nitric oxide (NO), and the activation of kinases for a differing physiological response (Deroo *et al.* 2006).

Estrogen is made up of three chemically different structures (figure 5); estrone (E1), estradiol (E2) and estriol (E3) (Coelingh Bennink 2004).

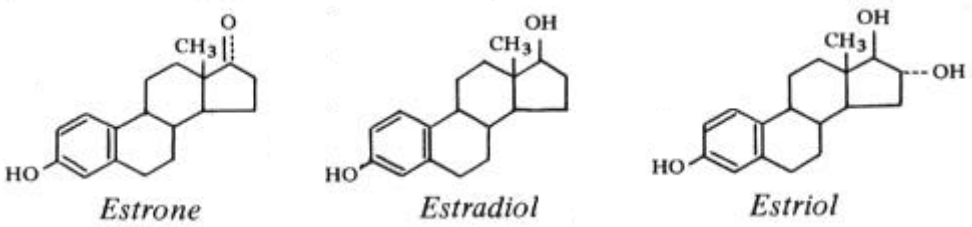

Figure 5: Chemical structure of estrone (E1), estradiol (E2) and estriol (E3) (www.worldofmolecules.com/emotions/estrogen.htm).

All three chemical forms of estrogen are produced naturally and are vital for growth, differentiation and development (Marino *et al.* 2006). Estradiol is the most estrogenic and has a greater ability to induce DNA damage in comparison to the less-estrogenic compounds, estrone and estriol (Yager *et al.* 1996). Estrogens are synthesised, metabolised, detoxified and excreted by a highly regulated system of enzymes (Olson *et al.* 2007; Hirata *et al.* 2008). To initiate estrogen metabolism, estradiol must be hydroxylated which mainly occurs on the carbon 2 (C2), carbon 4 (C4) or carbon 16 (C16) position of estradiol (Yager *et al.* 1996). Previous studies have

shown that C2 hydroxylation of estradiol (2-OH-E_2) is beneficial for cells as most of the cell proliferative and estrogenic activity is lost, thereby preventing cancer initiation (Schneider *et al.* 1984; Vandewalle *et al.* 1989). Conversely, hydroxylation of estradiol on C4 or C16 (4- or 16-OH-E2) has greater activity than estradiol alone and it has been shown that these metabolites are associated with genotoxicity and carcinogenesis (Barnea *et al.* 1983; Yager *et al.* 1996).

1.4.2 Progesterone

It has been well established that insufficient amounts of progesterone in the endometrium contributes to endometrial carcinogenesis as it functions to antagonise the toxic effects of estrogen (Graham *et al.* 1997; Pijnenborg *et al.* 2005).

Progesterone (figure 6) is a C-21 steroid hormone that is secreted by the ovary, is essential for female reproductive processes (menstrual cycle, pregnancy and targeting tissues such as ovaries and mammary glands) and limits cellular growth in the uterine epithelium (Martin *et al.* 1973). Progesterone is the precursor of the mineralocorticoid aldosterone, glucocorticoids (through conversion of progesterone to 17-hydroxyprogesterone) and androstenedione. Androstenedione can also be converted to testosterone, estrone and estradiol.

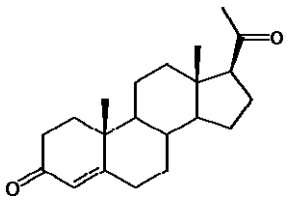

Figure 6: Chemical structure of progesterone (http://www.icgeb.trieste.it/~p450srv/ligand/progesterone.html).

The growth of the endometrium is controlled by the progesterone receptor. Progesterone binds to its receptor and antagonises the proliferative activity of estrogens, occurring through the initiation of differentiation of endometrial epithelium cells (Martin *et al.* 1973).

1.5 Risk Factors Associated with the Development of Endometrial Cancer

Excessive exogenous and/or endogenous estrogen exposure or insufficient progesterone to counter balance the effects of estrogen are determinants for endometrial cancer development. The environmental and reproductive risk factors related to endometrial cancer have been well established and are listed below (table 1

and reviewed by Bakkum-Gamez *et al.* 2008). These factors are also important for other hormone related cancers such as breast cancer (Harvey *et al.* 1985; Bakkum-Gamez *et al.* 2008).

Table 1: Exogenous and Endogenous Endometrial Cancer Risk Factors adapted from Bakkum-Gamez *et al.* 2008

Risk Factor	Influence of Risk Factor
Age	Greater risk of developing endometrial cancer as you age.
Early Menstruation	Longer exposure to estrogen
Late Menopause	
Hyperplasia	Precursor of endometrial cancer
Hormone Replacement Therapy (HRT)/Estrogen Replacement Therapy (ERT)	HRT does not initiate endometrial cancer development because of the presence of progesterone however ERT only has estrogen and increases the risk of developing endometrial cancer
Diabetes	Usually associated with obesity
High Blood Pressure (HBP)	
Obesity	Adipose tissue is directly related to an increase in circulating levels of estrogen and obese women have more adipose tissue
Tamoxifen	Breast cancer antagonist but endometrial cancer agonist
Race	Endometrial cancer is more common in Caucasians but African-American women have worse prognosis
Parity	The body produces more progesterone during pregnancy
Smoking	Smoking has been associated with earlier age of menopause and lower BMI which decreases circulating levels of estrogen and endometrial cancer risk
Polycystic Ovarian Syndrome	Hormonal imbalance that prevents ovulation and menstruation, thereby increasing endometrial risk
History of cancer	Cancers of the breast, ovary or bowel are associated with an increased risk of developing endometrial cancer
Hereditary non-polyposis colorectal cancer (HNPCC)	Hereditary condition - most common cancer in women with this syndrome is endometrial cancer

These exogenous and endogenous environmental and reproductive risk factors have the ability to alter the effects of estrogen in the endometrium and thereby contribute significantly to disease risk. Even though estrogen is the most influential determinant of endometrial cancer, it is important to note that a small percentage of endometrial cancers are caused by estrogen independent mechanisms.

1.6 Estrogen Dependent and Independent Endometrial Cancer

The majority of endometrial cancers can be divided into two broad categories; estrogen dependent and independent. This theory was first described by Bokhman in 1983 (Bokhman 1983) and an updated review is described by Ryan in 2005 (Ryan *et al.* 2005)

1. Estrogen Dependent Endometrial Cancer (Type I)

Estrogen dependent endometrial cancer (otherwise known as the unopposed estrogen hypothesis) is related to unopposed and prolonged estrogen stimulation. Approximately 80-90% of sporadic endometrial cancer cases are known as type I and are associated with endometrial hyperplasia, hyperestrogenism and expression of the estrogen receptors. These women can be pre or post menopausal, often have endometriod morphology and low grade carcinoma which is preceded by endometrial hyperplasia. Women with type I estrogen dependent disease generally have a good prognosis. Microsatellite Instability (MSI) and mutations in the genes, Kirsten sarcoma Retrovirus-Associated DNA Sequence (K-ras), beta-catenin and Phosphate and Tensin homolog (PTEN), are a common feature of this subtype (Ryan *et al.* 2005).

2. Estrogen Independent Endometrial Cancer (Type II)

Estrogen independent endometrial cancer accounts for approximately 10-20% of all sporadic endometrial carcinomas. It is unrelated to estrogen and displays negative or low estrogen receptor expression. These carcinomas have serous papillary or clear cell morphology and only post-menopausal women are diagnosed with this subtype. Type II independent endometrial cancer is not usually preceded by hyperplasia but the carcinomas are often high grade, and consequently have a poor prognosis. Mutations in human epidermal growth factor receptor type II (HER2/neu) and TP53 are commonly associated with this subtype (Ryan *et al.* 2005).

Therefore, type I and II endometrial carcinomas have significantly different genetic alterations and resultant phenotypes. The pathways involved in these two categories are described below.

1.6.1 Genetic Alterations in Estrogen Dependent Endometrial Carcinoma

1.6.1.1 Microsatellite Instability (MSI)

Microsatellites are short segments of DNA that have a repeated sequence. They are also known as variable number tandem repeats (VNTRs) or simple sequence repeats (SSRs). They are widely dispersed throughout the genome, however due to their repetitive structure, microsatellites are prone to mutation due to slippage errors that occur during DNA replication (Prat *et al.* 2007). Mutations that occur during DNA replication are corrected by a number of DNA repair processes. One of these repair pathways is DNA mismatch repair (MMR), comprising of 9 proteins that are involved in the recognition and initiation of DNA mismatch repair (Chao *et al.* 2006). If these processes are compromised, the MMR pathway does not initiate and microsatellite instability (MSI) arises (Thibodeau *et al.* 1993). MSI is commonly associated with hereditary non-polyposis colorectal cancer (HNPCC) syndrome; however, MSI has also been reported in sporadic cancers, including endometrial and colorectal cancer (Ichikawa *et al.* 1999).

Although endometrial cancer is the most common malignancy in women with HNPCC, approximately 20-30% of sporadic endometrial carcinomas display MSI (Risinger *et al.* 1993; Duggan *et al.* 1994; Caduff *et al.* 1996; Catasus *et al.* 1998). Mutation analysis following MSI testing has not revealed a high frequency of MMR mutations in endometrial cancer, suggesting that other genes or mechanisms of gene silencing are involved in cancer development (Katabuchi *et al.* 1995). One of these mechanisms is epigenetic silencing of hMLH1 due to hypermethylation, where the hMLH1 gene is inactivated in sporadic cancers (Herman *et al.* 1998). With respect to endometrial cancer, MSI is far more common in type I endometrial cancer compared to type II disease, 20-35% and 0-11%, respectively (Risinger *et al.* 1993; Duggan *et al.* 1994; Caduff *et al.* 1996; Catasus *et al.* 1998). It has been suggested that hMLH1 promoter hypermethylation is an early event in endometrial cancer since it has been associated with endometrial hyperplasia (Esteller *et al.* 1998).

1.6.1.2 K-ras

Kirsten sarcoma retrovirus-associated DNA sequence (K-ras) is a member of the ras family of genes that are a group of proteins harbouring GTPase activity. Ras genes control cell growth and differentiation, and function as the key in signalling pathways between cell surface receptors and the nucleus (Liu 2007; Prat *et al.* 2007). Enomoto *et al.* (1991) and Lagarda *et al.* (2001) examined mutations in K-ras and risk

of developing endometrial cancer and found that 10-30% of endometrial cancer cases had mutations in K-ras (Enomoto *et al.* 1991; Lagarda *et al.* 2001). Additionally, Lax *et al.* (2000) reported that variations found in K-ras are more common in type I endometrial cancers and tumours that are MSI positive, than in type II and MSI negative endometrial cancers (Lax *et al.* 2000). Further to this, two more recent studies have reported similar findings regarding mutations in K-ras and MSI, and the association with endometrial cancer (Lagarda *et al.* 2001; Prat *et al.* 2007). Interestingly, a study by Sasaki *et al.* (1993) examined K-ras variation in endometrial hyperplasia and found a similar frequency of mutations in comparison to women with endometrial cancer. From this, they suggested that K-ras could possibly be an early event in type I endometrial cancer initiation (Sasaki *et al.* 1993).

1.6.1.3 Beta-catenin

Beta-catenin is a submembranous protein encoded by the catenin (cadherin-associated protein) beta 1 (CTNNB1) gene. It has two major but independent roles: 1. Linking cytoskeleton actin filaments to transmembrane E-cadherin. 2. Important in the Wnt signal transduction pathway which is controlled by the adenomatous polyposis coli (APC) gene (reviewed by Moreno-Bueno *et al.* 2002). The accumulation of beta-catenin within the cells is usually removed by ubiquitin associated pathways. However, mutated beta-catenin does not undergo the processes of degradation and consequently accumulates within the cells, affecting the signal transduction pathway (Doll *et al.* 2008). Given that beta-catenin is essential for tissue maintenance, perturbations in beta-catenin may lead to cancer development. Mutations in beta-catenin account for 14-44% of endometriod cancers (Fukuchi *et al.* 1998; Saegusa *et al.* 2001; Scholten *et al.* 2003). Expression of beta-catenin in endometrial carcinoma is more common in type I than in type II endometrial carcinoma; 31-47% and 0-3%, respectively, and type I endometriod carcinomas with beta-catenin mutations are frequently early stage tumours, generally having a favourable prognosis (Moreno-Bueno *et al.* 2002; Schlosshauer *et al.* 2002).

1.6.1.4 PTEN

Phosphate and tensin homolog (PTEN) is a tumour suppressor gene which is located on chromosome 10q23.3. It encodes for a phosphatase that facilitates cell signal transduction pathways by acting on phospholipid phosphatidylinositol-(3,4,5)-triphosphate (PIP3) (Liu 2007). The PTEN phosphatase antagonises the PI3K/AKT pathway through the dephosphorylation of PIP3 and the decreased activity of this pathway increases cell growth and survival (Cully *et al.* 2006). This region is known in

many cancers to be a hot-spot for loss of heterozygosity (LOH) (Ali *et al.* 1999). PTEN mutations are found in 34-83% of type I endometriod carcinomas (Risinger *et al.* 1997; Mutter *et al.* 2000) and are more often found in patients that are MSI positive than MSI negative; 60-86% and 24-35% (Levine *et al.* 1998; Maxwell *et al.* 1998; Bilbao *et al.* 2006). In addition, PTEN mutations are present in 15-55% of endometrial hyperplasias, suggesting that PTEN alterations are an early event in carcinogenesis (Latta *et al.* 2002).

1.6.2 Genetic Alterations in Estrogen Independent Endometrial Carcinomas

1.6.2.1 HER2/neu

The human epidermal growth factor receptor type II (HER2/neu) oncogene is a transmembrane receptor tyrosine kinase (Liu 2007). It plays an important role in the organisation of the complex ErbB signalling network which is responsible for regulating the growth and differentiation of cells (Dougall *et al.* 1994; Graus-Porta *et al.* 1997). Upon activation, there is an increase in mitogen activated protein kinase and phosphoinositide-3 kinase (PI3K) cell signalling, leading to increased cell proliferation. However, high levels of HER-2/neu expression are associated with endometrial carcinomas that have been shown to be more aggressive due to chemotherapy resistance and consequently have poor survival (Prat *et al.* 2007). In 9-40% of all endometrial cancers, HER2/neu overexpression or amplification was reported and has been linked to decreased survival (Prat *et al.* 1994; Niederacher *et al.* 1999). Serous carcinomas, which are type II non-endometriod carcinomas, often display HER2/neu overexpression and have the lowest overall survival of all endometrial tumours (Slomovitz *et al.* 2004).

1.6.2.2 TP53

The TP53 (p53) tumour suppressor gene encodes for a nuclear protein that is important for the maintenance of genomic integrity by promoting apoptosis, through the Bax and Apaf-1 proteins (Yin *et al.* 1999). When DNA damage is recognised in the cell, p53 accumulates and the cell cycle is halted by p21. Consequently, p21 inhibits the phosphorylation of the retinoblastoma (Rb) gene and activation of cyclin D1 ultimately prevents propagation of damaged cells (Yin *et al.* 1999). Loss of p53 function can lead to numerous problems for genomic stability such as loss of heterozygosity (LOH), allelic imbalance and aneuploidy (Prat *et al.* 1994; Yin *et al.* 1999). Immunohistochemical analysis of p53 in non-endometriod carcinomas (type II endometrial cancer) revealed overexpression in 71-85% of cases (Ambros *et al.* 1996;

Kounelis *et al.* 2000). Additionally, about 20% of high grade endometriod carcinomas harbour p53 variants (Lax *et al.* 2000). Often mutations in p53 are associated with high grade tumours that have an advanced stage and aggressive histology, leading to an unfavourable prognosis (Semczuk *et al.* 2005). These results suggest that p53 is a late molecular event in type II endometrial cancers (Lax *et al.* 2000).

1.7 Genetic Alterations in Hereditary and Sporadic Endometrial Cancer

Many familial endometrial cancers are associated with the autosomal dominant inherited syndrome, hereditary non-polyposis colorectal cancer (HNPCC) (Boyd 2005). Outside of the context of HNPCC, approximately 5-8% of endometrial cancers are thought to arise due to a familial site-specific predisposition (Gruber *et al.* 1996; Sandles 1998; Ollikainen *et al.* 2005); however the literature concerning this topic is limited and warrants further investigation.

HNPCC, also known as Lynch Syndrome, is associated with a familial predisposition to colorectal cancer, endometrial cancer and a number of other extracolonic cancers (Lynch *et al.* 1993). It is characterised by early age of disease onset, neoplastic lesions, and germline variation in one of the mismatch repair genes; hMLH1, hMSH2, hMSH6, PMS2, causing microsatellite instability (MSI) (Lynch *et al.* 1999). The risk of developing endometrial cancer for women diagnosed with HNPCC is approximately 54%, similar to the risk of developing colorectal cancer at 52% (Hampel *et al.* 2005). Inactivation of the hMSH2/hMSH6 complex is more frequently associated with endometrial cancers in this syndrome (Schweizer *et al.* 2001; Hampel *et al.* 2005). Walsh *et al.* (2008) reported that one in five women diagnosed at an early age with endometrial cancer (less than 50 years) is expected to have a MMR gene mutation (Walsh *et al.* 2008).

Endometrial adenocarcinomas that are observed in HNPCC patients usually have endometriod histology, relating to type I endometrial cancer (Parc *et al.* 2000). However, mutations are generally not found in sporadic type I endometrial cancer patients, but instead the MMR genes (mainly hMLH1) are silenced or inactivated by promoter hypermethylation (Buttin *et al.* 2004). This phenomenon is not seen in type II endometrial cancer patients (Risinger *et al.* 2003). The highest risk of developing HNPCC related endometrial cancer is between the ages of 40 to 60 and one report showed that by the age of 70, 20% of HNPCC patients had developed endometrial cancer compared to only 3% in the general population (Watson *et al.* 1994). A study by

the same group identified that many of these women with endometrial cancer also had another primary tumour, most commonly colorectal cancer (Vasen *et al.* 1994).

A number of studies have suggested that some endometrial cancers are possibly related to an as yet unidentified gene due to the identification of familial site-specific endometrial cancers. One study reported that a small number of HNPCC families had a familial clustering of endometrial cancer in the absence of any other cancer type (Sandles 1998). Another group examined 23 site-specific endometrial cancer families and found two germline mutations in hMSH2 and hMSH6 (Ollikainen *et al.* 2005). Thus, only a very small percentage of familial site-specific endometrial cancer cases have been identified and it appears that these cases are most likely to be associated with HNPCC. Nonetheless, continued efforts are required to examine the underlying genetic determinants of familial endometrial cancer.

The majority of endometrial cancers are sporadic in nature and are likely to be associated with inter-individual genetic variation combined with related endometrial cancer risk factors. Many studies have shown that endometrial cancer aetiology is based upon excessive exposure to estrogen or exposure of estrogen in the absence of progesterone. The environmental factors that influence these hormones in hormone-responsive tissues are well known (table I) however determining the genetic factors related to endometrial cancer has only recently been recognised as an important avenue to explore. Studies that have focused on the molecular mechanisms involved in the tumour tissue of endometrial cancer patients have revealed a number of genetic alterations in the progression from hyperplasia to carcinoma in type I and II disease. However, the investigation of germline variants in these genes and their association with endometrial cancer has been limited. As a result, genetic variation underlying the initiation of endometrial cancer has not been well characterised but a number of reports (McGrath *et al.* 2006; Rebbeck *et al.* 2006; Hirata *et al.* 2008) have suggested that single nucleotide polymorphisms (SNPs) and other common variants in genes involved in hormone biosynthesis and the metabolism of these hormones could possibly play a significant role in disease initiation and progression.

1.8 Types of DNA sequence variations

Within the human genome there are a large number of subtle variations. These include single nucleotide polymorphisms (SNPs) and other types of polymorphisms: insertions, deletions, duplications, inversions, microsatellites and minisatellites (Wright 2005). The most common are single nucleotide polymorphisms (SNPs) and account for

a significant proportion of genetic variation in humans (Brookes 1999). SNPs are single nucleotide variations at a specific location in the genome that are present in more than 1% of the population (Collins *et al.* 1998; Sachidanandam *et al.* 2001). There are approximately 9 million SNPs identified to date with 1 occurring every 1000 base pairs (Suh *et al.* 2005). However, SNPs are not evenly distributed in the genome and they are less common in coding regions than in non-coding regions (Wang *et al.* 1998; Sachidanandam *et al.* 2001). SNPs located in non-coding regions are important for use as genetic or physical markers for comparative or evolutionary research studies. SNPs positioned in coding regions (accounting for 5% of the whole human genome) can affect transcription and the function of the encoded protein (Kim *et al.* 2007). SNPs found in coding regions can either be synonymous (no change in the amino acid due to the degenerate nature of the code) or non-synonymous (change in the amino acid) (Ramensky *et al.* 2002). Non-synonymous SNPs (nsSNPs) have the ability to alter the structure of the protein, thereby affecting protein function. nsSNPs are though to have a much greater impact than synonymous SNPs (sSNPs) and can cause disease or varied responses to certain stimuli (Ramensky *et al.* 2002).

The other genetic polymorphisms observed in humans; insertions, deletions, microsatellites and minisatellites, are widely dispersed throughout the human genome, however, they are mainly located in the non-coding regions, and therefore, are functionally neutral (Wright 2005). Similar to non-synonymous SNPs, a small percentage of these variants are not functionally neutral as they are present in the coding regions of the genome. They can affect gene expression and function, leading to genomic instability and the possible development of disease (Wright 2005).

Previous studies have suggested that polymorphic variation predates the divergence of ethnic groups and that variants displaying a high population frequency (SNPs >20%) are most likely to be shared by all populations whereas the variants that are specific for a population are likely to be rare and consequently difficult to find (Wright 2005). After the completion of the human genome, many researchers have started to investigate many different forms of genetic variation and the relationship between diseases in specific individuals (Suh *et al.* 2005). Most often, these association studies consist of a case-control population and examine a large number of polymorphisms in genes thought to be determinants of a particular phenotype (Dong *et al.* 2008). This highly utilised method is also referred to as the candidate gene approach where the results are generated through hypothesis testing.

The current study utilised the candidate gene approach to determine the association between endometrial cancer risk and polymorphisms located in genes of the following pathways: steroidogenesis, receptor activation, estrogen metabolism, cell cycle control and DNA repair. The current hypothesis is that polymorphisms rather than rare variants are the main determinants of genomic instability and it is essential to find these susceptibility genes in order to identify women at high risk of developing disease, and to improve patient treatment and management.

1.9 The Relationship between Hormone Biosynthesis and Endometrial Cancer

Cholesterol is the most abundant steroid in human mammalian cells. It has many different functions including influencing the structure and function of membranes, in addition to being a precursor of steroid sex hormones (Rog *et al.* 2008). The hormones that are synthesised from cholesterol and produced in the endocrine glands are estrogen, progesterone and testosterone. Five major genes are involved in the conversion of cholesterol to these hormones; CYP11A1, CYP17A1, CYP19A1, HSD17β1 and HSD17β2 (see figure 7) and variation in these genes may be implicated in the development of endometrial cancer.

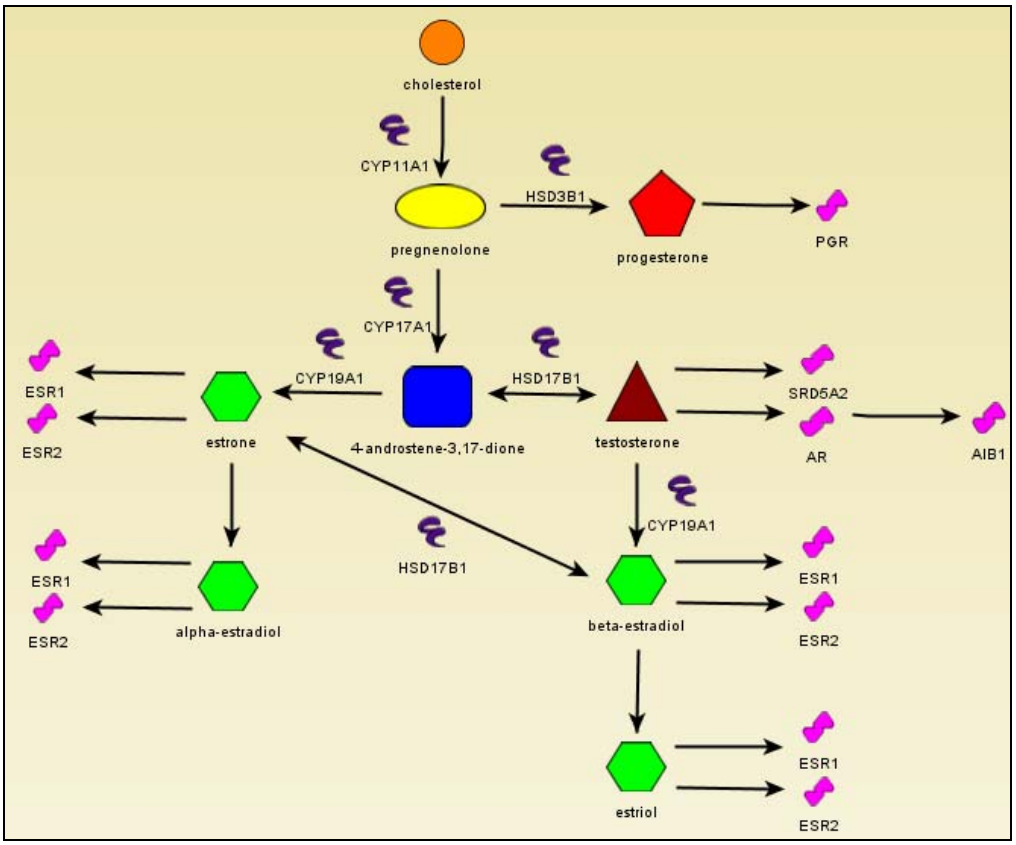

Figure 7: Steroidogenesis: estrogens (green), progesterone (red) and testosterone (dark red) are derived from cholesterol (orange) and by-products of cholesterol, pregnenolone (yellow) and androstenedione (blue). The enzymes that catalyse these chemical conversions are purple (CYP11A1, CYP17A1, CYP19A1, HSD17β1 and HSD3β1). The estrogen (ESR1 and ESR2), progesterone (PGR) and testosterone receptors (AR and SRD5A2) are pink.

1.9.1 CYP11A1

The cytochrome P450 11A1 gene (CYP11A1) is the first enzyme involved in steroidogenesis. The first rate limiting step of steroidogenesis involves the conversion of cholesterol to pregnenolone through the activity of the cholesterol side chain cleavage enzyme, CYP11A1.

To date, 58 SNPs have been identified in CYP11A1 (Olson *et al.* 2007). The pentanucleotide repeat (TTTTA)n polymorphism located at position -528 in the promoter region of the gene (D15S520) is the only variant that has been commonly studied in relation to cancer (Cicek *et al.* 2005; Ferk *et al.* 2006; Wang *et al.* 2006; Yaspan *et al.* 2007). However, this polymorphism has not been studied in women with endometrial cancer. Functional studies have not been performed on this repeat polymorphism and the majority of reports have shown that it has no association with altered levels of estrogen, progesterone or androgen hormones in premenopausal women (Gharani *et al.* 1997; Diamanti-Kandarakis *et al.* 2000; Daneshmand *et al.*

2002; Garcia-Closas *et al.* 2002). It is important however to determine the functional significance of this polymorphism as the promoter region contains multiple cAMP-regulated elements that are responsible for increasing basal transcriptional activity (Pestell *et al.* 1993; Gharani *et al.* 1997).

1.9.2 CYP17A1

The next step in hormone biosynthesis is the conversion of pregnenolone to 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA). These are precursors of testosterone and estrogen and this conversion is catalysed by the enzyme, CYP17A1. This enzyme is also involved in the conversion of progesterone to 17α-hydroxyprogesterone and androstenedione. The enzymes that perform these reactions are 17α-hydroxylase and C17,20 lyase, respectively and are products of cytochrome P450 17A1 (CYP17A1).

As with CYP11A1, many SNPs have been identified in CYP17A1. The most commonly examined SNP is a T>C variation 34bp upstream from the start codon in the 5' promoter region, however the functional impact of this SNP is unknown (Carey *et al.* 1994; Nedelcheva Kristensen *et al.* 1999). There is conflicting evidence in relation to the association of this variant and hormone levels in pre and postmenopausal women (Olson *et al.* 2007) though several association studies performed on this SNP and risk of endometrial cancer have reported inconsistent findings; some showed decreased endometrial cancer risk with the C allele and the other reports did not reveal any significant associations (Haiman *et al.* 2001; McKean-Cowdin *et al.* 2001; Berstein *et al.* 2004; Aban *et al.* 2006; Szyllo *et al.* 2006; Gaudet *et al.* 2008; Hirata *et al.* 2008).

1.9.3 CYP19A1

Cytochrome P450 19A1 (CYP19A1) encodes for aromatase, the rate-limiting step in the production of estrogen. It converts testosterone to estradiol in ovarian granulosa cells and androstenedione to estrone in adipose tissue (Olson *et al.* 2007). The most common polymorphisms examined in endometrial cancer are the (TTTA)n repeat in intron 4 and the 3 bp deletion (TCT) (rs11575899). Berstein *et al.* (2004) and Paynter *et al.* (2005) found an increased risk of developing endometrial cancer with greater than 7 repeats of the (TTTA)n polymorphism (Berstein *et al.* 2004; Paynter *et al.* 2005). Conversely, there has been no significant associations reported for the 3 bp deletion (Paynter *et al.* 2005).

1.9.4 HSD17β1 and HSD17βII

17β-hydroxysteroid dehydrogenase I and II are involved in the reduction and oxidation of estrogens to more and less active forms of estrogen, estradiol and estrone respectively. No association studies have been performed for variants in HSD17β2 however the G313S non-synonymous polymorphism in HSD17β1 has been studied in endometrial cancer although no significant findings were observed (Setiawan *et al.* 2004). Sequence homology analysis indicates that this SNP does not alter the functional significance of the protein (Johnson *et al.* 2005), adding further support towards the notion that this SNP is not associated with endometrial cancer risk.

1.10 The Relationship between Hormone Receptors and Endometrial Cancer

Hormones induce cellular changes and initiate their physiological effects through a number of different mechanisms. The most essential is the recognition and binding of the hormone to its receptor (estrogen to estrogen receptor α and β, progesterone to progesterone receptor, and testosterone to androgen receptor and its coactivator receptor, AIB1). Since these hormones are widely involved in many human tissues, it is highly likely that genetic variation in the receptors may be implicated in the initiation, development or progression of endometrial cancer.

1.10.1 Estrogen Receptor α and β (ESR1 and ESR2)

The effects of estrogens are mediated via binding to estrogen receptors (ESRs); estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2), resulting in the activation of a number of co-repressors and co-activators involved in its metabolism (Katzenellenbogen *et al.* 2000; Katzenellenbogen *et al.* 2000). The genes encoding ESR1 and ESR2 have similar structure and share substantial homology in DNA-binding and ligand-binding domains (Ogawa *et al.* 1998).

Genetic variation in the ESR genes can potentially result in ESRs with altered binding kinetics that adversely affect cellular metabolism. There is evidence to suggest that two ESR1 polymorphisms; rs2234693 (PvuII) and rs9340799 (XbaI), two dinucleotide ESR1 repeat polymorphisms; (GT)n and (TA)n and an ESR2 polymorphism; rs944050, affect receptor function due to differential splicing of the mRNA transcript (del Senno *et al.* 1992; Yaich *et al.* 1992; Roodi *et al.* 1995; Sand *et al.* 2002; Iwamoto *et al.* 2003; Beleza-Meireles *et al.* 2006). RNA stability of the ESR2 transcript is also considered to be affected by two other ESR2 polymorphisms; rs1255998 and rs4986938 located in the 3' untranslated region of the gene (Maguire *et*

al. 2005; Sowers *et al.* 2006). The ESR2 polymorphism rs1256049 in exon 5 causes a synonymous change of unknown functional significance (Setiawan *et al.* 2004).

A small number of studies have shown that polymorphisms in the ESRs alter the risk of developing endometrial cancer. The first case-control study conducted in Sweden showed a trend towards a decreased cancer risk in women with the variant ESR1 rs9340799 and rs2234693 genotypes and an increased cancer risk in women with the short alleles (<19 TA repeats) for the (TA)n dinucleotide repeat (Weiderpass *et al.* 2000). Similar findings were observed for the two single nucleotide polymorphisms in a Japanese case-control study (Iwamoto *et al.* 2003). However, a second Japanese study that examined the rs2234693 polymorphism alone did not confirm these findings (Sasaki *et al.* 2002). With respect to polymorphisms in ESR2, no associations of the rs1256049 and rs1271572 polymorphisms with endometrial cancer risk were found in a recent case-control study among Caucasians (Setiawan *et al.* 2004).

1.10.2 Progesterone Receptor (PGR)

Progesterone binds to the progesterone receptor (PGR) to initiate hormone signalling pathways. There are 2 isoforms of PGR: PR-A and PR-B. The main difference between the isoforms is that PR-B has 164 more amino acids at the N-terminal region of the gene. The isoforms are identical, but their actions are quite different (Kastner *et al.* 1990). PR-B is an activator of transcription whereas PR-A inhibits the function of PR-B and a number of other nuclear receptor genes, such as the estrogen receptors (Vegeto *et al.* 1993). The expression of both PGR isoforms in the endometrium is regulated by estrogen and progesterone, and during the menstrual cycle, a large change in the amount of these hormones takes place (Mote *et al.* 2000).

A polymorphism in the PGR, PROGINS, has been reported to increase endometrial cancer risk by decreasing response to progesterone (Romano *et al.* 2007). PROGINS is a 306bp Alu insertion in intron 7 and is in complete linkage disequilibrium with two other polymorphisms in exon 4 and 5, V660L and H770H, respectively. These polymorphisms serve as markers for the PROGINS polymorphism (McKenna *et al.* 1995; Agoulnik *et al.* 2004). Moreover, another two polymorphisms in PGR (+331G>A and +44T>C) have been identified in the promoter region and may affect relative transcription of PR-A and PR-B isoforms. Specifically, the +331G>A polymorphism favours increased transcription of PR-B (De Vivo *et al.* 2004). PR-B contributes to epithelial cell proliferation in response to estrogen alone and in the presence of both estrogen and progesterone. Therefore, PR-A expression is vital to oppose estrogen

induction and PR-B dependent proliferation (Mulac-Jericevic *et al.* 2000; De Vivo *et al.* 2002). Less is known about the +44T>C variant which has a low minor allele frequency (MAF) in Caucasians however since it is located in the promoter region, it may affect transcription. In regards to the +331G>A polymorphism, four studies have been conducted. De Vivo *et al.* (2002) found an increased risk of endometrial cancer with the +331G>A polymorphism (De Vivo *et al.* 2002); however Rebbeck *et al.* (2006) and Dossus *et al.* (2006) did not confirm this association (Dossus *et al.* 2006; Rebbeck *et al.* 2006). A study by Pijnenborg *et al.* (2005) examined the +331G>A and PROGINS polymorphisms in primary tissue of 44 patients with recurrent endometrial cancer (Pijnenborg *et al.* 2005). They found that the variant genotypes of the PROGINS polymorphism were related to a high risk of recurrent disease in those that had PR-expressing tumours (Pijnenborg *et al.* 2005).

One study has examined the association between the genomic DNA of women with endometrial cancer and the PROGINS polymorphism (Junqueira *et al.* 2007). They found that this polymorphism was associated with an increased risk of developing endometrial cancer since this polymorphism is thought to lead to a malfunction of the receptors, in turn leading to the accumulation of high levels of unopposed estrogen and allowing for increased cellular proliferation and possible cancer development (Junqueira *et al.* 2007). PROGINS has been shown to increase the stability of PR-A. This polymorphism is located in the hinge region which may affect bending of the chromatin and consequently, the activity of the enzyme (Metivier *et al.* 2003). The higher transcriptional activity may lead to inadequate control of PR-B and estrogen receptor alpha, leading to an increase in disease risk however these theories remain to be confirmed (Pijnenborg *et al.* 2005).

1.10.3 Androgen Receptor (AR)

Located on chromosome X, the androgen receptor is a member of the nuclear receptor subfamily of steroid receptors and functions as a transcription factor to regulate the transactivation of hormone-responsive genes (Tilley *et al.* 1989). It forms a dimer when testosterone or testosterone derivatives bind to the receptor. The AR then binds to androgen response elements (AREs) in androgen responsive genes (such as coactivators or corepressors), up-regulating their transcription (Ferro *et al.* 2002).

A highly polymorphic trinucleotide repeat of CAG is located in the first exon of the AR gene and encodes a polyglutamine tract in the N-terminal transactivation region (Ferro *et al.* 2002). Previous studies have demonstrated that shorter CAG repeats of

the AR are related with higher transcriptional activity (Chamberlain *et al.* 1994; Choong *et al.* 1996). Perutz *et al.* (1994) demonstrated that a higher number of repeats is associated with pathogenicity since the protein has a different structure and a reduced ability to recruit androgen responsive genes (Perutz *et al.* 1994), however the exact function of this repeat polymorphism is not known. In relation to endometrial cancer risk and the AR repeat polymorphism, six studies have been performed. A small number of studies have shown that a greater number of CAG repeats were associated with an increase in endometrial cancer risk (Yaron *et al.* 2001; Sasaki *et al.* 2003). Conversely, a study by McGrath *et al.* (2006) found the opposite association where increasing number of repeats were related to a decreased risk of developing endometrial cancer (McGrath *et al.* 2006). Another study showed that short CAG or GGN repeat polymorphisms in the AR are linked with benign endometrial cancer conditions in comparison to more invasive tumours (Rodriguez *et al.* 2006). Moreover, a study performed by Ju *et al.* (2007) found no association of age of diagnosis of endometrial cancer with the CAG repeat polymorphism (Ju *et al.* 2007), however the sample size was significantly less than those previously reported.

The results from the two association studies displaying an increased risk of endometrial cancer risk with increasing CAG repeat polymorphism number are in concordance with a study on BRCA1 breast cancer cases and early onset of breast cancer where the long CAG repeat length is related to an increased cancer risk (Rebbeck *et al.* 1999) and suggests that androgen is likely to exert an anti-mitogenic effect. A study performed by McGrath *et al.* 2006 reported that increasing repeat number is associated with decreased cancer risk which is in concordance with a prostate cancer study whereby androgen has a mitogenic effect and cancer risk increases with decreasing CAG repeat length (Ferro *et al.* 2002; McGrath *et al.* 2006). Due to the conflicting results and inconstancy between different cancer types, further analysis is required.

1.10.4 Amplified in Breast Cancer 1 (AIB1)

The amplified in breast cancer 1 (AIB1) gene is an AIB1/SRC-3 steroid hormone receptor coactivator member in the p160 family and can modulate the activity of the estrogen receptor and the progesterone receptor (Sakaguchi *et al.* 2007). A CAG/CAA repeat length polymorphism harbours 17 to 29 glutamines in the HR interacting carboxyl-terminal region of the protein (Shirazi *et al.* 1998). Thenot *et al.* (1999) demonstrated that over-expression of AIB1 mRNA was found in only MCF7 cells (breast cancer cell line) and not in any endometrial cancer cell line (Thenot *et al.*

1999). In 25 endometrial cancer patients with high AIB1 expression, their survival was poor in comparison to 15 endometrial cancer patients with low AIB1 expression (survival 36% and 96%, respectively) (Sakaguchi *et al.* 2007). Another study also found similar findings with high AIB1 expression being associated with poor prognosis of endometrial carcinoma (Balmer *et al.* 2006). In addition, they also suggested that co regulators may affect expression in the estrogen receptor, therefore high expression of AIB1 could alter the estrogen receptor, leading to the dysfunction of the receptor and possible development of cancer (Balmer *et al.* 2006).

The CAG/CAA repeat polymorphism in AIB1 has not been considered in endometrial cancer pathogenesis to date. Considering the previous reports showing altered expression of AIB1 in endometrial cancer cell lines and that AIB1 interacts with the estrogen receptor, it is possible that the repeat polymorphism may be implicated in the development of endometrial cancer. This polymorphism has been studied in other hormonally related diseases however the results have yielded inconsistent associations (Hsing *et al.* 2002; Li *et al.* 2005; Spurdle *et al.* 2006).

1.11 The Relationship between Estrogen Metabolism and Endometrial Cancer

It is well known that estrogens elicit a wide range of biological effects by binding to the estrogen receptor to induce the expression of genes involved in many important functions. Further to this, estrogens are eventually detoxified and eliminated by a complex network of metabolising enzymes. The efficiency of this process is essential as excessive or unopposed estrogen exposure is the main risk factor for endometrial cancer development. The pathways that are involved in the metabolism, detoxification and bioavailability of estrogen consist of a large number of enzymes, see figure 8. Genetic variations in these enzymes have been proposed to alter endometrial cancer risk. Estrogen undergoes two phases of metabolism: Phase I, estrogens are activated by biosynthetic enzymes and phase II, estrogens are detoxified by conjugation enzymes (Nebert *et al.* 2006).

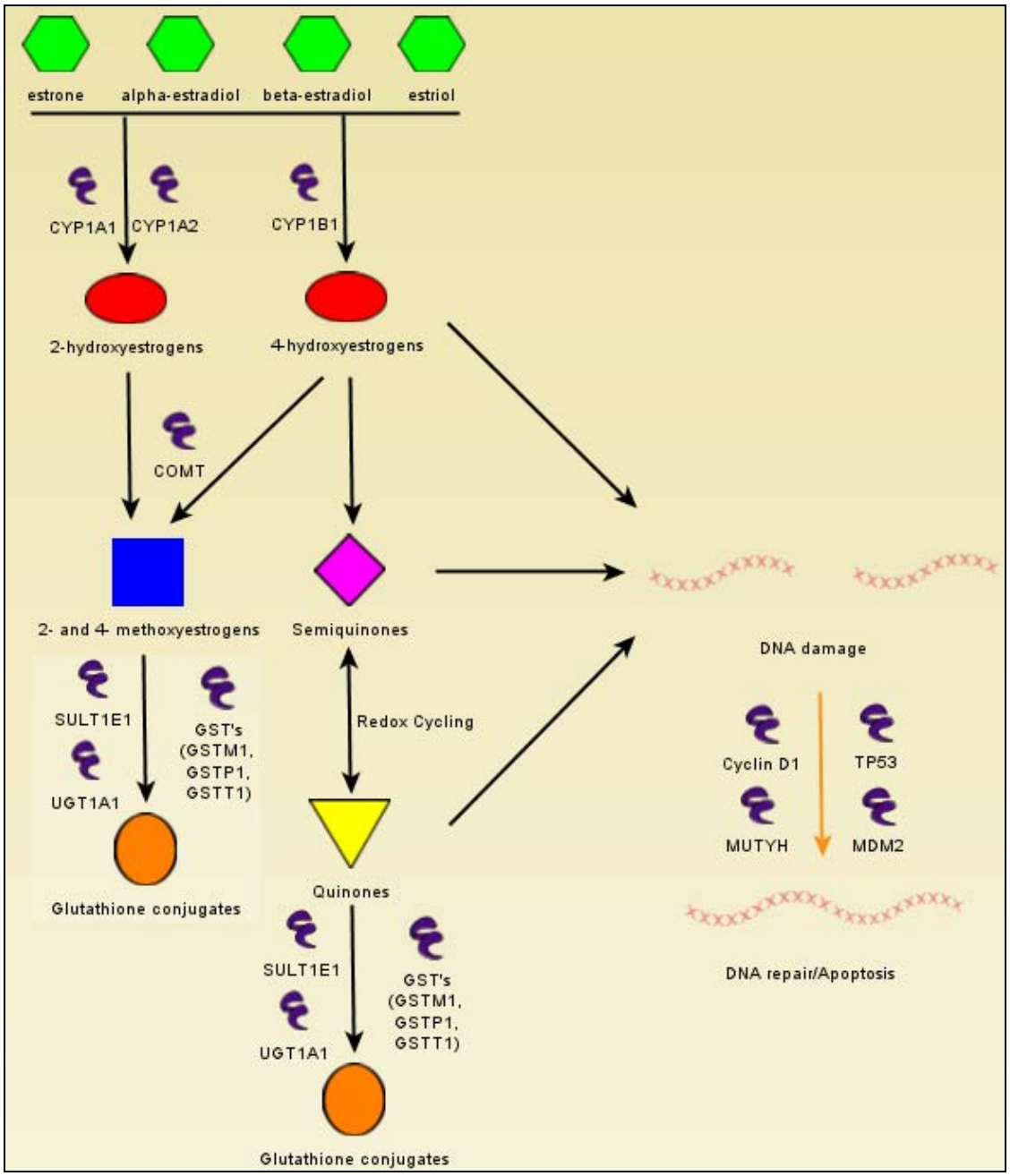

Figure 8: Estrogens, alpha and beta estradiol, estrone and estriol (green) are converted into 2- and 4- hydroxyestrogens (red) by the CYP1A1, CYP1A2 and CYP1B1 enzymes (purple). These metabolites are further broken down into biologically inactive 2- and 4- hydroxyestrogens (blue) by COMT (purple). 4-hydroxyestrogens that undergo redox cycling, forming semiquinones-quinones (pink and yellow) and 2- and 4- methoxyestrogens are detoxified by SULT1E1, GST's and UGT1A1 (purple) into glutathione conjugates (orange) for elimination. 4-hydroxyestrogens, semiquinones and quinones can cause DNA damage. Four enzymes, cyclin D1, MUTYH, TP53 and MDM2 (purple) involved in DNA repair and cell cycle control, either eliminate or repair DNA damage that has occurred.

1.11.1 Phase I Estrogen Metabolism

The first step of estrogen metabolism involves the conversion of estrogens into various catechol estrogen metabolites (2- and 4-hydroxyestrogens (2- and 4-OH-E_2) by the enzymes CYP1A1, CYP1A2 and CYP1B1.

1.11.1.1 CYP1A1

Cytochrome P450 1A1 (CYP1A1) catalyses the first oxidative step of estrogen (2-OH hydroxylation) in the endometrium (Tsuchiya *et al.* 2005). It is located on chromosome 15q24.1 and is expressed mainly in extrahepatic tissues. 2-hydroxylation of estrogens has been shown to inhibit tumour cell proliferation and appears to have anti-angiogenic effects (Zhu *et al.* 1998; Mooberry 2003).

Three CYP1A1 polymorphic variants exist in Caucasians: M1, M2 and M4. The M3 variant in intron 7 is specific for African-Americans (Crofts *et al.* 1993). The M1 variant in the 3' non-coding end of the gene contains a T>C base change which is essential for translational efficiency and mRNA stability. The M2 variant, I462V in exon 7, is a missense SNP that is near an active site in CYP1A1. The M4 variant, T461N is located adjacent to M2 in the hemebinding region but its functional significance is not known (Hirata *et al.* 2008). Some studies have reported that the M1 and M2 SNPs are involved in CYP1A1 inducibility and increased enzyme activity (Petersen *et al.* 1991). The relationship between these variants in CYP1A1 and endometrial cancer risk appear to indicate that they are associated with an increased risk of disease. A 3- to 4 fold increase risk in disease was attributed to the M1 and M2 polymorphisms (Esteller *et al.* 1997). In an extended study by the same group, examining the M4 polymorphism, a 6 fold increase in disease risk was observed (Esteller *et al.* 1997). In concordance with the findings for M2, Esinler *et al.* (2006) found that patients carrying the variant genotypes had an increased risk of endometrial cancer in a Turkish population (Esinler *et al.* 2006). Doherty *et al.* (2005) studied variants in CYP1A1, CYP1A2 and CYP1B1 and found in haplotype analysis that women carrying four or five low risk genotypes had a decreased risk of endometrial cancer (Doherty *et al.* 2005). Additionally, this group observed a decreased risk of developing endometrial cancer with the CYP1A1 M1 and M2 polymorphisms, but not the M4 polymorphism. Four case-control studies performed in the United States, Poland, Japan and Russia reported no significant relationship between these polymorphisms and cancer risk (Sugawara *et al.* 2003; Seremak-Mrozikiewicz *et al.* 2005; Mikhailova *et al.* 2006; McGrath *et al.* 2007).

1.11.1.2 CYP1A2

Cytochrome P450 1A2 (CYP1A2) is also involved in catalysing the first oxidative step of estrogen hydroxylation. This process occurs in the liver (Tsuchiya *et al.* 2005) and accounts for approximately 15% of cytochrome P450 content (Shimada *et al.* 1994). It plays a major part in the metabolism of a number of drugs (caffeine, phenacetin, clozapine, theophylline and paracetamol) and is involved in the activation

of several toxic compounds such as heterocyclic and aromatic amines (Tsuchiya *et al.* 2005).

The major role of CYP1A2 is to convert approximately 40-50% of estrogens into 2-hydroxyestrogens which are the anti-tumorigenic metabolites since they weakly bind to the estrogen receptors (Yamazaki *et al.* 1998). A polymorphism in intron 1 known as CYP1A2*1F (-163C>A) has been shown to increase CYP1A2 activity in Caucasians (Sachse *et al.* 1999). A study by Mikhailova *et al.* (2006) showed a positive association between the C genotype and an increase in hormone-dependent endometrial cancer risk in a Russian population (Mikhailova *et al.* 2006), where the C allele showed decreased enzymatic activity. In contrast, two larger studies did not confirm this finding (Rebbeck *et al.* 2006; Hirata *et al.* 2008). Haplotype analysis of CYP1A2 with CYP1A1 and CYP1B1 revealed that women with 4 or 5 low risk genotypes were at a decreased risk of developing endometrial cancer (Doherty *et al.* 2005).

1.11.1.3 CYP1B1

Cytochrome P450 1B1 (CYP1B1) is a polycyclic aromatic hydrocarbon (PAH) metabolising cytochrome. It activates carcinogens (such as N-nitrosamines found in tobacco) into reactive metabolites that have the ability to induce DNA damage (Newbold *et al.* 2000). As stated earlier, CYP1A1 and CYP1A2 are involved in the 2-OH of estrogens however CYP1B1 converts estrogens into 4-OH (Tsuchiya *et al.* 2005). This compound is highly carcinogenic and it has the ability to activate estrogen receptor alpha (Zhu *et al.* 1998). Four common polymorphisms exist in CYP1B1, R48G, A119S, L432V and N453S which have recently been shown to have approximately 2.4 to 3.4 times higher catalytic activity, leading to higher levels of 4-hydroxyestrogens (Hanna *et al.* 2000; Aklillu *et al.* 2002).

Doherty *et al.* (2005) studied variants in CYP1A1, CYP1A2 and CYP1B1 and risk of developing endometrial cancer. Haplotype analysis revealed that women carrying four or five low risk genotypes, conferred a decreased risk of endometrial cancer while risk for endometrial cancer increased when carrying the Val genotype of the L432V polymorphism (Doherty *et al.* 2005). Four studies showed no association of the V432L polymorphism in CYP1B1 and endometrial cancer susceptibility (McGrath *et al.* 2004; Rebbeck *et al.* 2006; Tao *et al.* 2006; Hirata *et al.* 2008). Sasaki *et al.* (2003) showed a 2.5 fold increase in risk in a Japanese population for the Val/Val genotype of CYP1B1 M2 polymorphism (Sasaki *et al.* 2003). In regards to another polymorphism in CYP1B1, N453S, one study revealed that carriers of the Ser allele were at a decreased

risk for developing endometrial cancer (McGrath *et al.* 2004). Additionally, a study on a Japanese population found an association for the A119S polymorphism. Endometrial cancer patients had a higher frequency of the variant genotypes which increased risk 3.32 times. The codon 449 and R48G polymorphisms, however, showed no correlations with endometrial cancer risk (Sasaki *et al.* 2003).

1.11.2 Phase II Estrogen Metabolism

The catechol estrogens (2- and 4-OH-E_2) created during phase I estrogen metabolism are eliminated by a number of processes in phase II estrogen metabolism: methylation (COMT), detoxification (GSTs), sulfination (SULT1E1) and glucoronidation (UGT1A1) (Zhu *et al.* 1998) (see figure 5).

1.11.2.1 COMT

The catechol estrogens formed by CYP1A1, CYP1A2 and CYP1B1 are inactivated and conjugated by catechol o-methyltransferase (COMT) to prevent quinone formation and redox cycling. COMT catalyses the reaction of catechol estrogens to form methoxy derivatives (2- and 4-methoxyestrogens, 2- and 4- MeOE_2), which are inactive by-products of catechol estrogens. The formation of 2- MeOE_2 is important as it has antiproliferative, cytotoxic and apoptotic activity (Dawling *et al.* 2001). Dawling *et al.* (2003) reported that the methoxy derivatives have a negative feedback function with CYP1A1, CYP1A2 and CYP1B1 inhibiting their function (Dawling *et al.* 2001). A functional polymorphism in COMT, V158M, has lower enzymatic activity for the Met genotypes compared to the Val genotypes (Zhu 2002). A number of association studies have been performed on the COMT V158M polymorphism and its relationship with endometrial cancer, however, no significant findings have been reported (McGrath *et al.* 2004; Doherty *et al.* 2005; Rebbeck *et al.* 2006; Tao *et al.* 2006; Hirata *et al.* 2008; Hirata *et al.* 2008).

1.11.2.2 Glutathione S Transferases (GSTs)

Glutathione S transferases (GSTs) are phase II enzymes involved in the conjugation of a wide range of electrophilic substances with glutathione, thus facilitating detoxification and further metabolism and excretion of carcinogens derived from estrogen. Through the conjugation of exogenous and endogenous compounds with electrophilic functional groups, the GSTs neutralise their electrophilic sites and create water soluble products (Hayes *et al.* 2005; Kala *et al.* 2007). GSTs are expressed in the endometrium (Barnette *et al.* 1999) and there are four main classes of GSTs: A, M, P, and T. Three important polymorphisms in GSTM1, GSTT1 and GSTP1 exist

however previous studies have not elucidated which GSTs are involved in the detoxification of reactive quinones and semiquinones (Roodi *et al.* 2004).

One report showed that GSTP1 is able to perform these functions however due to the overlapping structure of the different GSTs, it is likely that they all have some role in detoxification (Hachey *et al.* 2003). In GSTM1 and GSTT1 a partial deletion present in both genes results in a complete absence of functional activity of these enzymes (Garte *et al.* 2001). In GSTP1, the I105V polymorphism lies in close proximity to an electrophile (substrate) binding site. The Val variant is associated with increased or decreased activity depending on the specific substrate however generally this polymorphism causes decreased activity (Zimniak *et al.* 1994; Hu *et al.* 1997; Watson *et al.* 1998). Three reports have been published on the association between endometrial cancer and the GST polymorphisms mentioned above. Esteller *et al.* (1997) found a trend for an increased risk of endometrial cancer with the GSTM1 null genotype (Esteller *et al.* 1997) and Doherty *et al.* (2005) showed that an increase in endometrial cancer risk was associated with the GSTT1 polymorphism (Doherty *et al.* 2005). For GSTP1, a study found that endometrial cancer cases had a higher frequency of the variant Val genotypes in comparison to a control population, OR 1.59 (1.13-2.23) (Chan *et al.* 2005).

1.11.2.3 SULT1E1

Sulfotransferases (SULT) are a superfamily of cytosolic proteins which metabolise estrone, estradiol and catecholestrogens. They generate hydrophilic sulfates of estrogen that are excreted in the urine. Sulfation of these chemicals may protect cells from mitogenic and DNA damaging activities (Nowell *et al.* 2000; Adjei *et al.* 2003). The main genes involved in sulfation are SULT1A1 and SULT1E1. Expression of SULT1E1 is high within the endometrium and two recent studies reported an association between the G>A SNP at -64 and endometrial cancer susceptibility (Rebbeck *et al.* 2006; Hirata *et al.* 2008). The function of this variant is currently unknown and Rebbeck *et al.* (2006) suggest that this result could be due to a type I statistical error or the polymorphism could possibly be in linkage disequilibrium with another as yet unidentified variant (Rebbeck *et al.* 2006). SULT1A1 contains a common polymorphism R213H. The variant has been associated with a reduced rate of sulfotransferase activity (Ozawa *et al.* 1998) and has been implicated in a number of different hormonal cancers.

1.11.2.4 UGT1A1

Uridine diphospho-glucuronosyltransferase 1A1 (UGT1A1) is a member of phase II estrogen metabolism. It is required for the maintenance of hormone homeostasis in a number of steroid hormone response tissues (Belanger *et al.* 1998). The isozymes convert small lipophilic molecules (hormones, bilirubin, steroids and drugs) into excretable water soluble compounds by the covalent linkage to glucuronic acid (Burchell *et al.* 1998). Glucoronidation is presumed to block the oxidation of reactive metabolites (catechol estrogens) to quinones, thereby eliminating the carcinogenic effects of redox cycling (Belanger *et al.* 1998). A common polymorphism of two extra TA nucleotides in UGT1A1 is located in the TATA box in the promoter region (rs8175347). Increased numbers of repeats (more than the common 6 TA repeats) is associated with a 30% decreased transcription rate (Guillemette *et al.* 2001). Additional studies based on phenotypic measurements showed that women with 6/7 genotype had much lower glucuronidation rates compared to homozygous 7/7 carriers (Biondi *et al.* 1999; Iyer *et al.* 1999). Deming *et al.* (2008) have recently shown that the promoter polymorphism has no significant association with endometrial cancer risk which is in concordance with the Rebbeck *et al.* (2006) study (Rebbeck *et al.* 2006; Deming *et al.* 2008).

1.12 The Relationship between Testosterone Metabolism and Endometrial Cancer

In post-menopausal women, the main source of hormones is derived from the adrenal cortex (Vermeulen 1976). Many studies have examined the levels of adrenal hormones such as cortisol, androstenedione and dehydroepiandrosterone in women with endometrial cancer, reviewed by Akhmedkhanov *et al.* 2001. The reports have been conflicting though some have found increased levels of these hormones in women with endometrial cancer compared to a control population.

The most recent study, by Allen *et al.* 2008, examined circulating levels of endogenous sex hormones and risk of developing endometrial cancer. Interestingly, they found higher levels of circulating estrogens (estradiol, estrone and estriol) and free testosterone were associated with an increased risk of endometrial cancer in post-menopausal women (Allen *et al.* 2008). They suggested that the relationship between higher circulating levels of free testosterone and endometrial cancer risk could be explained by the peripheral conversion of testosterone into estradiol (Allen *et al.* 2008). Tuckerman *et al.* (2000) suggested that testosterone does not have a direct stimulatory effect on endometrial cell proliferation (Tuckerman *et al.* 2000), therefore increased

levels of free testosterone is directly related to increased circulating levels of estradiol, a known risk factor for the development of endometrial cancer (Allen *et al.* 2008). Taken together, these results provide support for the notion that genetic variation in testosterone metabolism may be associated with endometrial cancer risk. An important gene involved in this process is SRD5A2.

1.12.1 5α-reductase II (SRD5A2)

The 5α-reductase II enzyme (SRD5A2) is involved in the conversion of testosterone to 5α-dihydrotestosterone (DHT), a more potent natural androgen. DHT is essential for normal growth and development of the prostate gland (Scariano *et al.* 2008). A valine to leucine amino acid change at position 89 in SRD5A2 (V89L) is associated with decreased enzymatic activity and lower androgen levels (Makridakis *et al.* 2000). It has been hypothesised that higher levels of the enzyme are linked to increased prostate cancer risk (Scariano *et al.* 2008). Interestingly, Asian men have been found to have a higher frequency of the leucine genotypes in comparison to Caucasian men and the incidence of prostate cancer in Asian men is relatively lower (Makridakis *et al.* 1997; Febbo *et al.* 1999; Makridakis *et al.* 2000; Allen *et al.* 2001). A dinucleotide thymine-adenine repeat polymorphism (TA), located in the 3' untranslated region following exon 5, is hypothesised to be related to deregulation of SRD5A2 activity by the production of less stable mRNA transcripts (Thigpen *et al.* 1992). To date, no studies have been performed to elucidate the relationship between this polymorphism and endometrial cancer risk.

1.13 The Relationship between Genes Involved in DNA Repair and Cell Cycle Control, and Endometrial Cancer

Excessive or unopposed estrogen exposure is the main risk factor associated with endometrial cancer risk as it drives cell proliferation and consequently allows the opportunity for the accumulation of random genetic errors. To maintain genomic integrity, many processes involved in cell cycle control and DNA repair are essential for the recognition, initiation and repair or elimination of damage that occurs to the cells of the endometrium. There are a large number of genes involved in these processes; however, the focus of this study was to examine polymorphisms in four genes that are thought to be involved in altering endometrial cancer risk. These are: Cyclin D1, MUTYH, TP53 and MDM2.

1.13.1 Cyclin D1

Cyclin D1 (CCND1) is a key protein in the regulation of the cell cycle at the G1 to S phase transition, and is essential for regulation of proliferation, differentiation and transcriptional control (Sherr 1996). Overexpression of cyclin D1 induces excessive cellular proliferation and is a feature of a number of cancers, including endometrial and colorectal cancer (Arber *et al.* 1996; Ruhul Quddus *et al.* 2002; Choudhury *et al.* 2007; Horree *et al.* 2007; Watanabe *et al.* 2007). Specifically for endometrial cancer, numerous studies have reported increased cellular proliferation co-existing with progressive derailment of cyclin D1, leading to the progression of hyperplasia to endometrial endometriod carcinoma (Smid-Koopman *et al.* 2000; Semczuk *et al.* 2004; Horree *et al.* 2008). Many association studies have focused their attention on the functionally significant 870 G>A polymorphism in cyclin D1 which creates two different splice variant transcripts (Betticher *et al.* 1995). The normal transcript encodes exon 5 which is essential for ubiquitin-mediated proteolysis whereas the other transcript lacks the destruction box in exon 5 and increases the half life of cyclin D1 (Betticher *et al.* 1995). The A allele of the 870 G>A polymorphism in cyclin D1 encodes the alternate transcript and increased levels of cyclin D1 are also evident in the heterozygous state (Betticher *et al.* 1995; Hosokawa *et al.* 1998).

Previous studies have reported inconsistent findings for the cyclin D1 polymorphism and a number of different cancers. With respect to endometrial cancer, there has been one published report on the association between the cyclin D1 870 G>A polymorphism and endometrial cancer risk in Korean women (Kang *et al.* 2005). Kang *et al.* (2005) reported that endometrial cancer patients with the AA genotype had an increased risk of disease compared to carriers of the GG genotype and the combination of the GG and GA genotypes, suggestive of a recessive model for the A allele (Kang *et al.* 2005).

1.13.2 MUTYH

MUTYH (MYH) is a DNA glycosylase which plays an essential role in the base excision repair (BER) pathway to prevent the accumulation of mutations that are a result of oxidative DNA damage (Slupska *et al.* 1999). In 2002, two autosomal recessive inherited mutations in MUTYH, Y165C and G382D, were associated with adenomatous polyposis and colorectal cancer (Al-Tassan *et al.* 2002), and several additional studies have confirmed that bi-allelic mutation carriers have an increased risk of developing colorectal cancer (Croitoru *et al.* 2004; Farrington *et al.* 2005; Kairupan *et al.* 2005). These two mutations are reported to account for approximately

86% of all variations in the MUTYH gene that are identified in Caucasians (Barnetson *et al.* 2007).

Some studies have suggested that mono-allelic changes in MUTYH increase colorectal cancer risk however this remains to be confirmed (Croitoru *et al.* 2004; Farrington *et al.* 2005). Furthermore, association studies assessing mono-allelic changes in MUTYH in combination with DNA mismatch repair genes, has revealed a possible relationship, specifically between hMSH2 and hMSH6, and an increased risk of developing colorectal cancer although this remains to be definitively confirmed (Ashton *et al.* 2005; Niessen *et al.* 2006). Results from previous studies point towards a role of MUTYH mutations in HNPCC and suggest that they may be involved in a larger spectrum of disease involving extra-colonic cancers (Ashton *et al.* 2005; Niessen *et al.* 2006).

The role of MUTYH mutations in extra-colonic cancers has previously been reviewed and there are indications that this gene is associated with a broader spectrum of disease (Barnetson *et al.* 2007). The report by Barnetson *et al.* (2005) focused on determining whether variants in MUTYH were related to endometrial cancer risk (Barnetson *et al.* 2007). They identified one patient that was a compound heterozygote for Y165C and G382D. This patient had a sebaceous carcinoma which is a feature of Muir-Torre syndrome and is associated with MMR gene mutations. Five patients heterozygous for either Y165C or G382D were also identified. These MUTYH heterozygous mutation carriers did not harbour other pathogenic mutations, only a number of intronic variants. Given the conclusion that bi-allelic changes may increase susceptibility to endometrial cancer is based on one patient, these results need to be confirmed in a larger number of endometrial cancer cases.

In addition to the common MUTYH mutations, Y165C and G382D, three common polymorphisms in the Caucasian population have been identified: V22M, Q324H and S501F (Al-Tassan *et al.* 2002). These polymorphisms have been suggested as being associated with an increased risk of developing colorectal cancer, however, it remains to be determined if these changes are tissue specific with respect to disease risk. Notwithstanding, these five MUTYH variants represent a significant proportion of the genetic variation present in MUTYH and warrant further investigation.

1.13.3 TP53 and MDM2

One important pathway for the maintenance of genomic integrity involves the TP53 tumour suppressor gene and its negative regulator, mouse double-minute 2 homologue (MDM2). TP53 activation is induced in response to kinase signalling pathways that recognises DNA damage and it functions to regulate expression of genes involved in cell cycle arrest, apoptosis, DNA repair and angiogenesis to prevent the accumulation of genetic errors (Hollstein *et al.* 1991; Levine 1997; Soussi 2000). MDM2 has the ability to inactivate the function of TP53 through ubiquitinization and degradation, by direct binding to the protein (Chen *et al.* 1996; Kubbutat *et al.* 1997). MDM2 over-expression has been associated with many types of cancer where it has been shown to be involved in the inactivation of wild-type TP53 thereby obliterating cell cycle checkpoint control (Rayburn *et al.* 2005). Specifically for endometrial cancer, a direct relationship has been observed between increasing proliferation and progressive derailment of TP53 and MDM2 (Buchynska *et al.* 2007; Horree *et al.* 2008).

Numerous polymorphisms have been reported in TP53; however three appear to have functional effects that have been related to a change in malignant potential. These polymorphisms are R72P, a 16bp insertion in intron 3 and a G>A polymorphism in intron 6. Wu *et al.* (2002) performed functional studies on cell lines expressing at least one variant allele of the three polymorphisms and found that the ability of TP53 to regulate DNA repair processes was significantly reduced (Wu *et al.* 2002). The TP53 Arg (72R) allele is more prone to human papillomavirus oncoprotein (E6) mediated degradation than the Pro (72P) allele (Storey *et al.* 1998). Furthermore, the R72P polymorphism has been shown to alter the efficiency of p73, which is a TP53 homolog and transcription factor that responds to DNA damage and initiates apoptotic signalling pathways (Marin *et al.* 2000; Irwin *et al.* 2003). A polymorphism located in the promoter region of MDM2, SNP309, results in increased MDM2 levels and reduces the activity of TP53 (Bond *et al.* 2004). Since TP53 and MDM2 are central components in the maintenance of genomic integrity, these polymorphisms may be associated with endometrial cancer.

The TP53 R72P polymorphism has been studied in a number of cancers however association studies involving this polymorphism and endometrial cancer risk have shown varying results. The R72P polymorphism has been suggested to be in linkage disequilibrium with the 16bp insertion in intron 3 and the G>A polymorphism in intron 6. Ueda *et al.* (2006) studied the relationship between the R72P polymorphism and the risk of developing endometrial cancer and found an increased risk of disease in

patients harbouring the Arg/Arg genotype compared to those with combined Arg/Pro and Pro/Pro genotypes (Ueda *et al.* 2006). Conversely, a study by Roh *et al.* (2004) found an increased risk of endometrial cancer in carriers of the Pro allele (Roh *et al.* 2004). Both studies were conducted among Asian populations (Japanese and South Korean). Due to their small sizes (108 cases, 95 controls; 95 cases, 285 controls) the statistical power of these studies however was weak. Moreover, three studies reported no associations (Esteller *et al.* 1997; Peller *et al.* 1999; Niwa *et al.* 2005).

Furthermore, a study by Saffari *et al.* (2005) examined the relationship between twelve TP53 genetic alterations including the R72P polymorphism in 59 endometriod carcinomas and lower overall survival and responsiveness to adjuvant radiotherapy (Saffari *et al.* 2005). The R72P polymorphism was identified in seven of the twelve variants identified and women carrying the Arg/Pro genotype had a lower overall survival than those with the wild type Arg/Arg genotype. Additionally, women harbouring the Arg/Pro genotype who did not receive adjuvant radiation therapy had a significantly lower survival rate than those with the Arg/Pro genotype whom received treatment. Treated women with the Arg/Pro genotype had a similar survival rate to those women with the wild-type Arg/Arg genotype. These results suggested that women with the TP53 Arg/Pro genotype have an altered response to radiation induced DNA damage.

Recently, two reports have studied the association of the MDM2 SNP309 T>G polymorphism and the risk of developing endometrial cancer. The first report published by Walsh *et al.* (2007) found that women with the GG genotype were at a greater risk of developing endometrial cancer compared to those carrying the TT and TG genotypes (Walsh *et al.* 2007). This study involved a relatively small cohort (n=73 cases, n=79 controls), potentially reducing the power to detect true associations due to type I statistical error. A more recent Caucasian study on larger cohorts (Nurses Health Study: n=454 cases, n=1132 controls; Women's Health Study: n=137 cases, n=411 controls) provided further evidence for an increased risk of disease in GG carriers compared to TT carriers (Terry *et al.* 2008).

1.14 Summary

Inherited variation in genes involved in steroidogenesis, hormone receptors, estrogen metabolism, cell cycle control and DNA repair may be involved in altering the risk of developing endometrial cancer and is most likely influenced by lifetime exposure to exogenous and endogenous endometrial cancer risk factors. Notwithstanding, the identification of inter-individual variation in the genes involved in these pathways may aid in the detection of women at high risk of developing endometrial cancer to aid in better treatment and patient outcomes.

Aims

The focus of the work described in this thesis was to examine variants in genes involved in hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair and cell cycle control and their association with endometrial cancer risk, utilising the candidate gene approach.

This thesis is subdivided into six manuscripts:

I – The Association of the COMT V158M Polymorphism with Endometrial/Ovarian Cancer in HNPCC families adhering to the Amsterdam Criteria

II – Polymorphisms in genes of the steroid biosynthesis and metabolism pathways and endometrial cancer risk

III – Estrogen receptor polymorphisms and the risk of endometrial cancer

IV – Polymorphisms in TP53 and MDM2 combined are associated with high grade endometrial cancer

V – The influence of the Cyclin D1 870 G>A polymorphism as an endometrial cancer risk factor

VI – Genetic variants in MUTYH are not associated with endometrial cancer risk

STATEMENT I

This statement explains the contribution of all authors in the article listed below:

Ashton, K.A., Meldrum, C.J., McPhillips, M. L., Suchy, J., Kurzawski, G., Lubinski, J. and Scott, R. J. (2006) The Association of the COMT V158M polymorphism with Endometrial/Ovarian Cancer in HNPCC families adhering to the Amsterdam Criteria. *Hereditary Cancer Clin. Pract*. 4(2) pp. 94-102

Table I: Author Contribution Percentage and Description of Contribution to the Article Listed Above

Author	Contribution %	Description of Contribution to Article	
Katie A. Ashton	70%	Designed and executed the study. Provided significant insight into the interpretation of the data. Wrote the manuscript.	
Cliff Meldrum	4%	Supplied samples and clinical information.	
Mary McPhillips	4%	Contributed to technical assistance. Supplied samples and clinical information.	
Janina Suchy	4%	Supplied samples and clinical information.	
Grzegorz Kurzawski	4%	Supplied samples and clinical information.	
Jan Lubinski	4%	Supplied samples and clinical information. Established the Polish hereditary cancer centre – Szczecin.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected the manuscript.	

Chapter II

The Association of the COMT V158M polymorphism with Endometrial/Ovarian Cancer in HNPCC families adhering to the Amsterdam Criteria

Hereditary Cancer in Clinical Practice 2006; 4(2) pp. 94-102

The Association of the COMT V158M Polymorphism with Endometrial/Ovarian Cancer in HNPCC Families Adhering to the Amsterdam Criteria

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Key words: HNPCC, colorectal cancer, endometrial cancer, COMT V158M, MMR, mutations

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Abstract

Catechol-O-methyltransferase (COMT) is vital for the conjugation of catechol estrogens that are produced during oestrogen metabolism. The efficiency of this process varies due to a polymorphism in COMT, which changes valine to methionine (V158M). The Met genotypes slow the metabolism of catechol oestrogens, which are agents that are capable of causing DNA damage through the formation of DNA adducts and reactive oxygen species (ROS) production. The slower metabolism of catechol oestrogens results in there being a higher circulating concentration of these oeastrogens and consequently greater probability of DNA damage. To determine whether metabolic inefficiencies of oeastrogen metabolism are associated with the development of malignancy in hereditary non-polyposis colorectal cancer (HNPCC), we studied the V158M polymorphism in COMT in a large cohort of 498 HNPCC patients from Australia and Poland that were either mutation positive (n=331) or negative (n=167) for mismatch repair (MMR) gene mutations (hMLH1 or hMSH2). HNPCC is a familial predisposition to colorectal cancer (CRC) and extracolonic cancers that include endometrial cancer.

Using Real Time PCR, the COMT V158M polymorphism was examined and its association with disease expression, age of diagnosis of cancer, mutation status and mutation type was assessed in the HNPCC MMR mutation positive and negative groups. This study showed that the V158M polymorphism had no association with disease risk in the HNPCC MMR mutation positive population. However, the polymorphism was significantly associated with endometrial/ovarian cancer risk in HNPCC MMR mutation negative patients (p=0.002). The heterozygous (Val/Met) genotype was associated with an increased risk of developing endometrial/ovarian cancer whereas the homozygous mutant (Met/Met) showed a decreased risk. The results suggest heterosis, where there is an apparent greater effect of the heterozygous state in this dichotomous trait. In conclusion, this study shows that the COMT V158M polymorphism alters the risk of developing endometrial/ovarian cancer in patients that adhere to the Amsterdam HNPCC criteria but do not have a DNA mismatch repair gene mutation.

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant inherited disorder associated with a familial predisposition to colorectal cancer (CRC) and endometrial cancer (EC). It is characterised by early age of disease (CRC) onset, neoplastic lesions, microsatellite instability (MSI) and an increased incidence of extracolonic cancers [1]. Familial colorectal cancer syndromes, HNPCC and familial adenomatous polyposis (FAP), account for around 3% of all colorectal cancer cases; however, approximately 20% show familial inheritance of which there is no known genetic cause [2]. Mutations of the genes involved in the mismatch repair pathway, hMLH1 and hMSH2, account for a large proportion of patients that fit the HNPCC clinical criteria [1, 3]. Approximately 80% of men and 40% of women that have germline mutations in MMR genes develop CRC [1, 4] and 25-50% and 8-12% of women develop endometrial cancer and ovarian cancer, respectively [5]. While environmental factors are thought to play an important role in HNPCC disease aetiology, other as yet unknown genetic factors are also likely to contribute to HNPCC disease susceptibility, and it has been suggested that single nucleotide polymorphisms (SNPs) contribute to disease. Polymorphisms in genes involved in many biological pathways (DNA repair, xenobiotic clearance, immune response and a number of other pathways) have been examined, but the role of polymorphisms in oestrogen metabolism genes has not been characterised in the HNPCC population.

Catechol-O-methyltransferase (COMT) is a phase II enzyme involved in oestrogen metabolism. It catalyses the addition of a methyl group to catechol oestrogens and converts them into methoxy derivatives [6]. Catechol oestrogens are believed to contribute to oestrogen-induced cancer through their ability to initiate DNA damage by the formation of DNA adducts and reactive oxygen species (ROS) [7, 8]. 2-Methoxyestrone has a protective role in the development of cancer since it is antioestrogenic, inhibits tumour growth, stimulates apoptosis and inhibits angiogenesis [6-9]. Therefore, the conversion of catechol oestrogen into 2-methoxyestrone is important in the elimination of toxic agents by conjugation [10]. The highest COMT activity occurs within the brain, liver, kidney, endometrium and breast [11].

The first study examining the function of COMT revealed that its activity is low, intermediate or high [12]. The three levels of activity correspond to a trimodal distribution. In 1995, Lotta et al. [13] identified a G to A polymorphism in COMT, which results in a valine to methionine amino acid change at position 158 of the gene (known as V158M). COMT is polymorphic within the general population since approximately 50% have the intermediate and 25% have the low activity forms of the polymorphism [14]. The A allele is thought to be associated with a 4-5 times less efficient metabolism of oestradiol than the G allele [12], which subsequently allows the accumulation of higher circulating levels of oestradiol. Heterozygous individuals have intermediate COMT activity [14].

The V158M polymorphism has been studied in a variety of hormonally influenced cancers such as prostate [15], breast [16 and references within], ovarian [17, 18] and endometrial cancer [19, 20]. Some of these studies have found positive associations between the low activity allele and cancer risk but other studies have not found any association and in some cases the opposite association has been reported. For that reason, the role of V158M polymorphism in COMT and cancer risk remains unresolved.

To our knowledge there have been no studies examining the COMT V158M polymorphism and colorectal and endometrial cancer risk in HNPCC patients. So far, there have been only two studies that have looked at the V158M polymorphism and endometrial cancer and three studies involving colorectal cancer. A study by Doherty et al. [19] showed a modest decreased risk of developing endometrial cancer with the Met allele, which was not expected. Another study by McGrath et al. [20] found no association between endometrial cancer and the polymorphism. In addition, a study by Sasaki et al. [21] showed that promoter region of membrane bound COMT (MB-COMT) was methylated in 47/60 endometrial cancer tumours. Methylation of the promoter region silences the gene and they concluded that this may contribute to endometrial carcinogenesis. All of the studies that examined the polymorphism and colorectal cancer susceptibility showed no associations [22-24]. Also, Garner et al. [17] and Sellers et al. [18] studied ovarian cancer susceptibility and the COMT V158M polymorphism. Garner et al. [17] concluded that Val/Met variant of COMT decreases the risk for mucinous tumours, but both studies reported no other associations. In conclusion, the role of the V158M polymorphism in COMT and its relation to cancer have previously shown inconsistent findings.

Since endometrial cancer is the most common cancer in women that have HNPCC and the genetics of endometrial and ovarian cancer within the context

of HNPCC are poorly understood, it is important to identify other genes involved in susceptibility to disease. COMT is a good candidate as another gene involved in disease since COMT is highly expressed within the endometrium and previous studies have shown associations between the COMT polymorphism and oestrogen-influenced cancers. Additionally, the functioning of COMT is important for the degradation of catechol oestrogen and the conversion to 2-methoxyestrone to prevent the formation of DNA adducts and ROS. For this reason, the role of the V158M polymorphism in COMT is important to elucidate in the HNPCC population.

Materials and methods

Subjects

498 patients were included in this study. The patients were selected from across the state of New South Wales and from Poland because they fulfilled the clinical criteria of hereditary non-polyposis colorectal cancer (HNPCC). Approval for this study was obtained from the Hunter Area Research Ethics Committee (Australia), the University of Newcastle Human Research Ethics Committee (Australia) and the Ethics Committee of the Pomeranian Academy of Medicine (Poland). All patients enrolled in this study had given informed consent for their anonymous DNA to be used for research into genetic predispositions to colorectal cancer. Two HNPCC groups were examined: those with mutations in MMR genes, hMLH1 or hMSH2 (mutation positive – 331 patients) and those without mutations in these genes (mutation negative – 167 patients).

HNPCC MMR Mutation Positive Population

The selection criteria for the HNPCC MMR mutation positive group were based on the molecular diagnosis of HNPCC; 331 patients harboured a confirmed causative mutation in either hMLH1 or hMSH2, of which there were 285 nonsense, insertion, deletion or splice mutations (leading to a truncated protein) and 46 missense mutations described as pathogenic in the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) mutation database. There were two subpopulations of Caucasians in this study – Australian and Polish. In the Australian population, there were 197 samples collected in the state of New South Wales from 1998 to 2004, and in the Polish population there were 134 samples collected from 1997 to 2002. Of the 331 individuals, 149 had been diagnosed with colorectal cancer: 94 in the Australian population and 55 in the Polish population. Of the 197 Australian and 134 Polish patients, 107 (54%) and 78 (58%) were relatives of probands, respectively.

Population subgroups

To determine any association between the disease characteristics of the mutation positive group and the V158M polymorphism, the samples were subdivided into different subgroups according to: (i) gene mutation status (hMLH1 or hMSH2); (ii) mutation type: truncation/deletion (including insertion, deletion, nonsense and splice site changes) or pathogenic missense mutations and; (iii) disease expression (affected/unaffected with CRC or affected/unaffected with endometrial/ovarian cancer). The age of onset of CRC was defined as the patient's age at diagnosis, while the age of the unaffected patients was determined by subtracting their date of birth from their age at the time of testing. A subgroup of patients unaffected with CRC over the age of 45 years was prepared to compare with the patients affected with CRC. This was performed since patients under the age of 45 years whom are not affected with disease could possibly still develop disease later in life. The age of diagnosis of CRC was unknown for 7 Australian and 5 Polish patients, and the disease expression status was unknown for 4 Australian patients.

Combined HNPCC mutation positive populations (Australian and Polish)

The Australian and Polish populations were combined to determine any association between the disease characteristics of the mutation positive group and the V158M polymorphism. The analysis was performed in the same way as mentioned above (population subgroups).

HNPCC Mutation Negative Population

To determine if the V158M polymorphism is associated with disease expression in HNPCC mutation negative patients, the samples were divided into those affected with CRC and affected or unaffected with endometrial/ovarian cancer. The mutation negative population was previously tested to determine whether they harboured a germline mutation in the hMLH1 or hMSH2 genes by denaturing high performance liquid chromatography (dHPLC) analysis followed by direct sequencing, multiplex ligation probe amplification (MLPA) assay and denaturing gradient gel electrophoresis (DGGE). From all analyses performed, no

mutation was found in the samples. This group of patients was collected from 1997 to 2004. Within this group there were 167 Australian samples, all of which were affected with CRC or affected/unaffected with endometrial/ovarian cancer (155 affected with CRC, 21 affected with endometrial/ovarian cancer, 9 affected with both CRC and endometrial/ovarian cancer). Of the 167 patients, 6 (3.5%) were relatives or probands. The age of diagnosis of CRC was unknown for 2 patients.

DNA isolation

Genomic DNA was isolated from Na_2EDTA blood according to the method previously described by Miller et al. [25].

Real-time PCR SNP genotyping

DNA samples were genotyped to determine the allele frequency of the COMT V158M polymorphism. Allelic discrimination was performed on an ABI PRISM 7900HT sequencing detection system (PE Applied Biosystems, Foster City). Assay-by-Design℠, a service offered by Applied Biosystems (PE Applied Biosystems), was used to design primers and probes. The primers and probes used were 5″-CCCAGCGGATGGTGGAT-3″ (forward primer), 5″-CAGGCATGCACACCTTGTC-3″ (reverse primer), 5″-VIC-TTCGCTGGCATGAAG-3″ (wildtype probe) and 5″-FAM-TCGCTGGCGTGAAG-3″ (mutant probe). The assay functions under universal conditions with each reaction containing: 50 ng DNA, 0.125 μl 40× Assay Mix and 2.5 μl TaqMan Universal PCR master mix made up to 5 μl with sterile water. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 70 cycles of 92°C for 15 sec and 60°C for 1 min. Post PCR, the plate was scanned to allow discrimination between the different genotypes.

Statistical analysis

Statistical analysis was undertaken to assess whether or not the polymorphism segregates with specific types of disease expression, mutation type, mutation status or age of diagnosis of CRC in HNPCC patients. The Hardy-Weinberg equilibrium (HWE) was assessed for the subject groups. All statistical tests were performed on the statistical software package Intercooled Stata 8.0 (Stata Corporation, Texas) and Statistical Package for the Social Sciences (SPSS) 12.0 (SPSS, Chicago). The significance levels for all tests were set at $p<0.05$. The genotype distribution between the different groups was analysed using Pearson's chi-squared test and if the number of samples in a given group was less than 5, Fisher's exact test was used. Kaplan-Meier survival analysis was used to compare genotype and age of diagnosis of CRC. To assess the homogeneity of the survival curves the Wilcoxon, log rank and Tarone-Ware tests were used. The log rank p value was only reported when there were no significant results.

Results

HNPCC Mutation Positive Group

Disease expression in Australian HNPCC mutation positive patients compared to Polish patients

There was no significant difference in the frequency of affected and unaffected CRC patients observed between the two populations. The proportion of hMLH1 and hMSH2 carriers was similar in both populations, and the proportion of colorectal cancer patients was similar for hMLH1 and hMSH2 mutation carriers. The subgroup endometrial/ovarian cancer had a similar frequency of individuals affected and unaffected with CRC (11 affected and 12 unaffected in Australia and 5 affected and 9 unaffected in Poland). In addition there was no significant difference between the two populations in the frequency of truncation/deletion and missense mutations.

Allele frequency distribution of COMT V158M in Australian and Polish HNPCC mutation positive patients

The distribution of the V158M polymorphism in this study was in Hardy-Weinberg equilibrium (HWE) in both populations. The three genotypes in the V158M polymorphism were Val/Val (homozygous wildtype/GG), Val/Met (heterozygous/GA) and Met/Met (homozygous mutant/AA). There was a statistically significant difference in the allele frequency distribution of the polymorphism between the two populations ($p=0.02$). The Australian population had a higher frequency of the heterozygous genotype and a lower frequency of the homozygous mutant genotype compared to the Polish population. When the subject group was subdivided according to their gene mutation status there was a significant difference observed between the Australian and Polish hMLH1 mutation carriers ($p=0.03$), where the Australian patients had a much higher proportion of the Val/Met (GA) genotype and a much lower proportion of the Met/Met (AA) genotype. There was no statistical difference seen between the Australian and Polish hMSH2 mutation carriers. There was a statistically significant result

Table 1. Allele frequency distribution of the COMT V158M polymorphism in the Australian and Polish HNPCC MMR mutation positive patients

Group	Population	Val/Val (%)	Val/Met (%)	Met/Met (%)	n	Pearson's Chi-squared
subject group	Australia	53 (26.9)	108 (54.8)	36 (18.3)	197	p=0.02
	Poland	33 (24.6)	59 (44.0)	42 (31.3)	134	
hMLH1 mutation carriers	Australia	31 (28.7)	59 (54.6)	18 (16.7)	108	p=0.03
	Poland	17 (23.0)	32 (43.2)	25 (33.8)	74	
hMSH2 mutation carriers	Australia	22 (24.7)	49 (55.1)	18 (20.2)	89	p=0.41
	Poland	16 (26.7)	27 (45.0)	17 (28.3)	60	
mutation type: truncation/deletion	Australia	50 (28.2)	95 (53.7)	32 (18.1)	177	p=0.05
	Poland	26 (24.1)	49 (45.4)	33 (30.6)	108	
mutation type: missense	Australia	3 (15.0)	13 (65.0)	4 (20.0)	20	p=0.20
	Poland	7 (26.9)	10 (38.5)	9 (30.6)	26	
affected with CRC	Australia	24 (25.5)	51 (54.3)	19 (20.2)	94	p=0.88
	Poland	14 (25.5)	28 (50.9)	13 (23.6)	55	
unaffected with CRC	Australia	28 (28.3)	53 (53.5)	18 (18.2)	99	p=0.02
	Poland	19 (24.1)	31 (39.2)	29 (36.7)	79	
unaffected with CRC (>45 years)	Australia	10 (23.3)	24 (55.8)	9 (20.9)	43	P=0.84
	Poland	5 (22.7)	11 (50)	6 (27.2)	22	
endometrial/ovarian cancer	Australia	6 (26.1)	14 (60.9)	3 (13.0)	23	p=0.12
	Poland	5 (35.7)	4 (28.6)	5 (35.7)	14	
affected with CRC and unaffected	Australia	22 (22.7)	57 (58.8)	18 (18.6)	97	p=0.09
with endometrial/ovarian cancer	Poland*	28 (23.3)	55 (45.8)	37 (30.8)	120	

** This group contained males and females. The sex was unknown for all of the patients.*

involving the patients unaffected with CRC between the two populations (p=0.02). The Australian population had a higher frequency of the heterozygous genotype and a lower frequency of the homozygous mutant genotype in comparison to the Polish population (see Table 1).

Allele frequency distribution in the Australian and Polish groups analysed separately and combined

When assessing disease expression, mutation status and mutation type between the different subgroups in the two populations separately, there were no significant difference observed in the Australian and Polish populations. In addition there were no significant differences involving disease expression, mutation status and mutation type when the populations were combined (see Table 2).

Median age of diagnosis of CRC in the subject groups

The median age of diagnosis of CRC was similar in both populations, 42.5 years in the Australian group with a range from 17 to 70 years and 44 years in the Polish group with a range from 18 to 78 years. In individuals with hMLH1 and hMSH2 mutations the median age of diagnosis was 42 years for both in the Australia population, with a range from 17 to 64 years in hMLH1 mutation carriers and 22-76 years in hMSH2 mutation carriers. In the Polish population the median age of diagnosis was 45 years for hMLH1 mutation carriers (32-78 years) and 41 years for hMSH2 mutation carriers (18-78 years).

Kaplan-Meier survival analysis

There was no significant difference between genotype and age of diagnosis of CRC in the

Table 2. Allele frequency distribution of the COMT V158M polymorphism in the Australian and Polish HNPCC MMR mutation positive patients combined

Group	Val/Val (%)	Val/Met (%)	Met/Met (%)	n	Pearson's Chi-squared
subject group	86 (26.0)	167 (50.5)	78 (23.6)	331	
hMLH1 mutation carriers	48 (26.4)	91 (50.0)	43 (23.6)	182	p=0.98
hMSH2 mutation carriers	38 (25.5)	76 (51.0)	35 (23.5)	149	
mutation type: truncation/deletion	76 (26.7)	144 (50.5)	65 (22.8)	285	p=0.65
mutation type: missense	10 (21.7)	23 (50.0)	13 (28.3)	46	
affected with CRC	38 (25.5)	79 (53.0)	32 (21.5)	149	p=0.50
unaffected with CRC	47 (26.4)	84 (47.2)	47 (26.4)	178	
unaffected with CRC (>45 years)	15 (23.1)	35 (53.8)	15 (23.1)	65	p=0.92**
endometrial/ovarian cancer	11 (29.7)	18 (48.6)	8 (21.6)	37	p=0.66
affected with CRC and unaffected with endometrial/ovarian cancer*	50 (23.0)	112 (51.6)	55 (25.3)	217	

** This group contained males and females. The sex was unknown for all of the patients.*
*** This group was compared to patients affected with CRC*

Australian and Polish groups (Australian population p=0.19 and Polish population p=0.48). However, there was a trend observed in the Australian population where patients with the Met/Met genotype had a later age of onset of CRC compared to the other genotypes.

HNPCC Mutation Negative Group

Disease expression in Australian HNPCC mutation negative patients

The distribution of the V158M polymorphism was in Hardy-Weinberg equilibrium (HWE) in this population. There was a statistically significant difference in genotype frequency between patients affected with endometrial/ovarian cancer compared to those unaffected (p=0.002) (see Table 3). The endometrial/ovarian cancer group had a higher frequency of the heterozygous (GA) genotype and lower frequency of the other genotypes in comparison to the patients unaffected with endometrial/ovarian cancer (see Fig. 1).

Median age of diagnosis of CRC

The median age of diagnosis of CRC in the HNPCC MMR mutation negative group was 51 years with a range of 19 to 74 compared to 42.5 years with a range of 17 to 70 years in the mutation positive Australian group. The 8.5 years difference in median age was not statistically significant.

Kaplan-Meier survival analysis

There was no significant difference between genotype and age of diagnosis of CRC in the Australian mutation negative group (p=0.81).

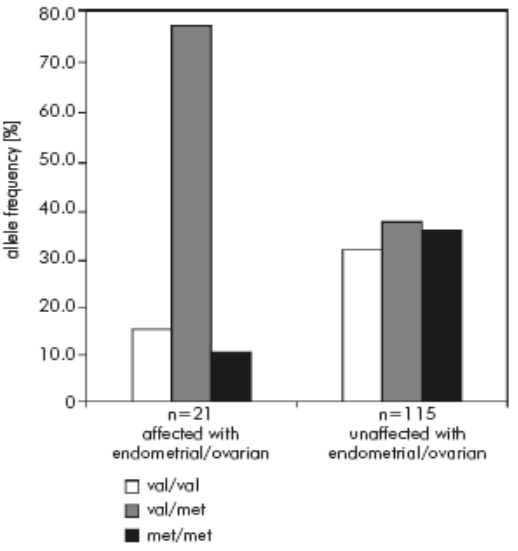

Fig. 1. Allele frequency distribution of the COMT V158M polymorphism in the Australian HNPCC MMR mutation negative patients assessed by endometrial/ovarian cancer disease expression

Table 3. Allele frequency distribution of the COMT V158M polymorphism in the Australian HNPCC MMR mutation negative patients

Group	Val/Val (%)	Val/Met (%)	Met/Met (%)	n	Pearson's Chi-squared
subject group	45 (26.9)	78 (46.7)	44 (26.4)	167	
endometrial/ovarian cancer	3 (14.3)	16 (76.2)	2 (9.5)	21	p=0.002
affected with CRC and unaffected with endometrial/ovarian cancer*	35 (30.4)	41 (35.7)	39 (33.9)	115	

* *This group contained females only.*

Discussion

There have been numerous candidate SNP studies performed in the past involving colorectal cancer and endometrial cancer, which have focused on a variety of biological pathways. The role of modifier genes in disease is becoming recognised as an important factor in understanding the variation that can be observed in individuals who harbour mutations in the same gene. In addition they could possibly account for a proportion of patients that do not harbour a mutation in a known gene, yet still fit the clinical characteristics of the syndrome.

In this study, we examined the V158M polymorphism in COMT to determine its association with colorectal cancer and endometrial/ovarian cancer in two different groups: an Australian and a Polish population that harboured a mutation in hMLH1 or hMSH2, and an Australian population that did not harbour a mutation in hMLH1 or hMSH2. COMT is involved in oestrogen metabolism and it functions to methylate catechol derivatives to render these carcinogens inactive [6]. Functional studies performed demonstrate that the Met allele hinders the metabolism of oestradiol and allows for greater levels of oestradiol to circulate [12]. One recent study showed that the Met allele has the same level of activity as the Val allele but has greater susceptibility to 4-hydroxyequilenin (4-OHEN) mediated inhibition and thermolability [26].

The frequency of the three genotypes Val/Val, Val/Met and Met/Met in the subject groups were 26.9%, 54.8% and 18.3% Australian MMR mutation positive, 24.6%, 44%, 31.3% Polish MMR mutation positive, and 27.0%, 46.7% and 26.3% in the Australian MMR mutation negative genotype frequencies. The Polish and Australian MMR mutation negative frequencies are in accordance with a number of Caucasian control groups [18, 27-29] and provide evidence that there is no difference in the allele frequencies between HNPCC patients in our study and other Caucasian populations. However, the genotype frequency in the Australian MMR mutation positive group was statistically significantly different to the Polish population in addition to the other control populations. This is most likely due to the high numbers of relatives involved in the study.

The significant differences observed in the mutation positive group when the Australian population was compared to the Polish population (gene mutation status, hMLH1 $p=0.03$ and disease expression, unaffected with CRC $p=0.02$) can be accounted for by the fact that this population is not random and contains a large proportion of proband relatives. When the Australian and Polish MMR mutation positive populations were either compared separately or as a combined group there were no significant differences in the frequency of the COMT polymorphism, which suggests that the V158M variant does not influence disease expression, gene mutation status, mutation type or age of diagnosis of CRC.

In the mutation negative group, a statistically significant difference was observed in relation to endometrial/ovarian cancer ($p=0.002$). Patients affected with endometrial/ovarian cancer had a higher frequency of the Val/Met genotype and a lower frequency of the other genotypes in comparison to those patients unaffected with endometrial/ovarian cancer. The samples used for this part of the study were from the Australian population only; therefore we believe the results to be representative of mutation negative HNPCC families from Australia, and it remains to be seen if these results are similar in other populations.

The significance of these results can be interpreted in two different ways. Firstly, the Val/Met genotype is known to cause intermediate activity of COMT and consequently it is less efficient in the detoxification of the oestrogen metabolites within the endometrium, which ultimately leads to carcinogenesis. Secondly, the low frequency of the Met/Met genotype in the endometrial/ovarian cancer group indicates a protective role of this genotype. The results suggest that endometrial/ovarian cancer susceptibility is more complex than the COMT V158M polymorphism and it is indicative that many other genes or other variants

within COMT will provide further insight into the mechanisms of disease in HNPCC. Li et al. [26] reported that the Ala22Ser polymorphism in COMT showed lower methylation capacity and higher thermolability and thus might be of functional importance in oestrogen-related cancers. These results are of interest since there has previously been no known genetic predisposition in patients that fit the clinical criteria for HNPCC who do not have a MMR gene mutation. COMT V158M therefore could account for some of the endometrial/ovarian cancer cases within the HNPCC population. Alternatively, this may be an example of heterosis [see review 30], where there is an apparent greater effect of the heterozygous state in this dichotomous trait.

Other studies performed involving the COMT V158M polymorphism and CRC, endometrial cancer and ovarian cancer have thus far shown no strong associations with disease. Doherty et al. [19] showed a weak association with the variant alleles displaying a protective role for the development of endometrial cancer. Our results are in accordance with those since the Met/Met genotype in this study appears to be protective for endometrial cancer; however, in this study the heterozygous genotype appears to be causative of disease. Our results regarding CRC risk and V158M are in accordance with the three previous studies performed which showed no association; therefore we conclude that it is highly unlikely that COMT affects CRC risk in HNPCC patients [22-24].

Several limitations in our study warrant caution in the interpretation of the findings presented. Firstly, the size of the subgroups was in some cases quite small and therefore lacked adequate power to detect a small increase in cancer risk. Multiple comparisons were performed to assess disease expression, mutation status and mutation type, which increased the risk of type one errors. Additionally, it is important for future studies to look at the functions of the examined variants and their associated genes to provide a clearer role of susceptibility to disease. The assessment of such interactions will be the focus of future analyses.

Although this study suggests that the heterozygous genotype and homozygous mutant genotype are causative and protective of endometrial/ovarian cancer respectively, these associations should be carefully interpreted and confirmed in a much larger population of women that fit the clinical criteria for HNPCC and have endometrial cancer in addition to a sporadic endometrial cancer population.

In conclusion, the variation observed in MMR gene mutation carriers in regards to disease expression, mutation type, mutation status and age of diagnosis of CRC in HNPCC families is not influenced by the COMT V158M polymorphism. It appears that the polymorphism might account for some of the endometrial/ovarian cancer cases observed in the HNPCC MMR mutation negative population and also in some patients confer a protective role for developing endometrial/ovarian cancer. It is likely that other modifying factors, both genetic and environmental, play a role in the variation in disease expression observed in HNPCC.

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STATEMENT II

This statement explains the contribution of all authors in the articles listed below:

Ashton, K.A., Proietto, A., Otton, G., Symonds, I., McEvoy, M., Attia, J., Gilbert, M., Hamann, U. and Scott, R.J. (2009) Polymorphisms in genes of the steroid biosynthesis and metabolism pathways and endometrial cancer risk. *(Submitted)*

Ashton, K.A., Proietto, A., Otton, G., Symonds, I., McEvoy, M., Attia, J., Gilbert, M., Hamann, U. and Scott, R.J. (2009) Estrogen receptor polymorphisms and the risk of endometrial cancer. *BJOG* Jul;116(8):1053-61

Ashton, K.A., Proietto, A., Otton, G., Symonds, I., McEvoy, M., Attia, J., Gilbert, M., Hamann, U. and Scott, R.J. (2009) Combined TP53 R72P and MDM2 SNP309 genotypes are associated with high grade endometrial cancer. *Gynecol. Oncol.* Apr;113(1):109-114

Ashton, K.A., Proietto, A., Otton, G., Symonds, I., McEvoy, M., Attia, J., Gilbert, M., Hamann, U. and Scott, R.J. (2008) The influence of the Cyclin D1 870 G>A polymorphism as an endometrial cancer risk factor. *BMC Cancer* Sept;29(8):272

Table II: Author Contribution Percentage and Description of Contribution to the Articles Listed Above

Author	Contribution %	Description of Contribution to Article	
Katie A. Ashton	70%	Designed and executed the study. Provided significant insight into the interpretation of the data. Wrote the manuscripts.	
Anthony Proietto	2.5%	Supplied case samples and clinical information.	
Geoffrey Otton	2.5%	Supplied case samples and clinical information.	
Mark McEvoy	2.5%	Supplied control samples and clinical information.	
Ian Symonds	2.5%	Contributed to the design of the study.	
John Attia	2.5%	Supplied control samples and clinical information. Assisted with statistical analysis.	
Michael Gilbert	2.5%	Contributed to technical assistance.	
Ute Hamann	5%	Contributed to the design of the study, provided technical assistance and assisted in the preparation of the manuscripts.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected all manuscripts.	

Chapter III

Polymorphisms in genes of the steroid hormone biosynthesis and metabolism pathways and endometrial cancer risk

Polymorphisms in genes of the steroid hormone biosynthesis and metabolism pathways and endometrial cancer risk

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Abstract

Background: The incidence of endometrial cancer has recently increased substantially and studies have shown that altered levels of exogenous and endogenous hormones are associated with individual variation in endometrial cancer risk. The environmental and reproductive risk factors that influence these hormones are well known, however, genetic variants involved in hormone biosynthesis and estrogen metabolism have not been well established in endometrial cancer.

Methods: To determine whether polymorphisms in genes of the steroid hormone biosynthesis and metabolism pathways are associated with endometrial cancer risk, we genotyped 28 polymorphisms in 18 genes in 191 endometrial cancer cases and 291 healthy controls.

Results: The GSTM1 deletion and the variant (GG) genotype of the CYP1B1 rs1800440 polymorphism were associated with a decreased risk of developing endometrial cancer. Furthermore, combinations of haplotypes in CYP1A1, CYP1B1 and GSTs were associated with a decreased risk. The analysis of the repeat polymorphisms revealed that women with the long repeat allele length of the ESR1 (GT)n repeat polymorphism were at an increased risk of developing endometrial cancer. Conversely, women with two long repeat length alleles of the (CAG)n repeat in the AR correlated with decreased endometrial cancer risk compared to women with one or two alleles with the short repeat length.

Conclusions: The findings are consistent with our hypothesis that variability in genes involved in the hormone receptors and estrogen metabolism may alter the risk of developing endometrial cancer, suggesting that they may be useful as biomarkers for genetic susceptibility to endometrial cancer.

Introduction

Endometrial cancer is the most common gynaecological cancer in the industrialised world. It has been well established that unopposed estrogen or excessive estrogen exposure is the main cause of disease (Akhmedkhanov *et al.* 2001). Recently, the incidence of endometrial cancer has increased substantially (AIHW 2007) and altered levels of exogenous and endogenous hormones have been directly related to differences in disease risk (Bakkum-Gamez *et al.* 2008).

Estrogens, progesterone and testosterone exert a variety of important physiological effects, which are mediated by their respective receptors, estrogen receptor alpha and beta (ESR1 and ESR2), progesterone receptor (PGR), the androgen receptor (AR) and their co-regulators. After the hormones have exerted their physiological effect, they are metabolised and excreted. Successful detoxification and elimination of estrogens is necessary to prevent prolonged or excessive exposure to estrogens and their metabolites (Zhu *et al.* 1998), and it is considered an important etiological factor for the induction of endometrial cancer .

The production of estrogens, progesterone and testosterone occurs through the steroidogenesis process where cholesterol is firstly synthesised into pregnenolone by the enzyme, cytochrome P450 11A1 (CYP11A1). Thereafter, progesterone is produced from pregnenolone by 3β-hydroxysteroid dehydrogenases (HSD3β1 and HSD3β2). Cytochrome P450 17A1 (CYP17A1) converts pregnenolone and progesterone into androstenedione, a precursor for estrogen and testosterone production. The enzymes, cytochrome P450 19A1 (CYP19A1) and hydroxysteroid dehydrogenases (HSD17β1 and HSD17β2), are involved in the production of estrogens (estrone, estradiol and estriol) and testosterone.

Estrogen metabolism and elimination involves two main steps, classified into two stages. Phase I involves the conversion of estrogen into catechol metabolites and hydroxy derivatives by the enzymes; cytochrome P450 1A1, 1A2 and 1B1 (CYP1A1, CYP1A2 and CYP1B1) through hydroxylation (Zhu *et al.* 1998). CYP1A1 and CYP1A2 catalyse the 2-hydroxylation of estrogens in the endometrium and liver, respectively (Weisz *et al.* 1992; Lee *et al.* 2003) and previous studies have shown that 2-hydroxylation is the main oxidative pathway in the endometrium and these estrogenic by-products prevent the formation of tumours (Zhu *et al.* 1998; Mooberry 2003). CYP1B1 catalyses the 4-hydroxylation of estrogens that are found in smaller quantities

than 2-hydroxyestrogens however they have been associated with the initiation of carcinomas in the endometrium (Liehr 2000). Additionally, 4-hydroxyestrogens can activate the estrogen receptor, thereby increasing the quantity of estrogen within the cells (Zhu *et al.* 1998). In endometrial cancer, the ratio between 4-hydroxylation and 2-hydroxylation increases proportionally with the inititiation of tumorigenesis (Weisz *et al.* 1992).

Phase II involves the inactivation and elimination of catechol estrogens by the processes of detoxification, oxidation, methylation, sulfination and glucuronidation (Zhu *et al.* 1998). The main enzymes involved in this process include: catechol O-methyltransferase (COMT), glutathione transferases (GSTs), sulfotransferases (SULTs) and uridine diphospho-glucuronosyltransferases (UGTs). Following the metabolic activation of estrogens (2- and 4- hydroxyestrogens), the catechol estrogens are inactivated by COMT (2- and 4-methoxyestrogens) or they are oxidised into quinones and semi-quinones (Cavalieri *et al.* 1997). These products have the ability to perform redox cycling and have been shown to have detrimental effects through the formation of DNA damage and production of oxidative stress (Yager 2000). Similarly to phase I estrogen metabolism, 2-methoxyestrogens do not induce DNA damaging events however 4-methoxyestrogens form depurinating DNA adducts which can occur in vital genes that control biosynthesis, metabolism and inactivation of estrogens (Zhu *et al.* 1998; Mooberry 2003; Doherty *et al.* 2005). Therefore, COMT is a key enzyme for preventing quinone and semiquinone formation via the methylation of hydroxyestrogens (Dawling *et al.* 2001). GSTs, SULTs and UGTs inactivate any quinones or semiquinones formed, ultimately lending to their elimination (Belanger *et al.* 1998; Nowell *et al.* 2000; Adjei *et al.* 2003; Hayes *et al.* 2005; Kala *et al.* 2007).

Given that unopposed estrogen and excessive estrogen exposure are the main risk factor associated with endometrial cancer, it is reasonable to hypothesise that interindividual variation in genes of hormone biosynthesis and estrogen metabolism pathways may increase the risk of developing disease. These genes have well characterised polymorphisms which have been studied previously in relation to hormonally related cancers including endometrial cancer. We investigated the association of endometrial cancer risk and polymorphisms in the following genes involved in hormone biosynthesis and metabolism: CYP11A1, CYP17A1, CYP19A1, HSD17B1, estrogen receptor alpha (ESR1), androgen receptor (AR), progesterone receptor (PGR), amplified in breast cancer I (AIB1), CYP1A1, CYP1A2, CYP1B1, COMT, GSTM1, GSTT1, GSTP1, SRD5A2, SULT1E1 and UGT1A1.

To determine if polymorphisms in genes involved in steroid hormone biosynthesis and metabolism are associated with endometrial cancer risk, we genotyped 28 polymorphisms in 191 endometrial cancer patients and 291 age and sex matched controls.

Material and Methods

Study Population

This study initially consisted of 213 consecutively recruited women with histologically confirmed endometrial cancer who presented for treatment at the Hunter Centre for Gynaecological Cancer, John Hunter Hospital, Newcastle, New South Wales, Australia between the years 1992 and 2005. Women that had additionally been diagnosed with breast cancer were excluded from this study.

The final analysis included 191 endometrial cancer patients. Data on reproductive and environmental risk factors including ethnicity, body mass index (BMI), diabetes, high blood pressure (HBP), age of diagnosis of endometrial cancer, age of menarche, age of menopause, other personal cancer history, family history of cancer (defined as cancer in the index patient plus one or more first or second degree relatives diagnosed with cancer), parity, breastfeeding, oral contraceptive use, chemotherapy, radiotherapy, hormone therapy (HT), smoking and alcohol use was collected using self reported questionnaires. Information regarding recurrence, stage, grade and histology of endometrial cancer was collected from the medical records.

The control population consisted of 291 participants that were recruited between the years 2004 and 2005 for the Hunter Community Study. This study aims to identify genetic and environmental factors associated with ageing in a cohort of individuals obtained from the Hunter region, Newcastle, New South Wales, Australia. Any control that had a prior diagnosis of either breast or endometrial cancer was excluded from the study. Controls were matched to cases by sex and age.

All participants provided informed written consent prior to participation in this study. Ethics approval was obtained from the Human Research Ethics Committee, University of Newcastle and the Hunter Area Research Ethics Committee, Hunter New England Health Service, Newcastle, New South Wales, Australia.

DNA Isolation

Genomic DNA was extracted from 10ml EDTA blood as previously described (Miller *et al.* 1988).

Molecular Analysis

A total of 28 variants in 18 genes were included in this study. The polymorphisms examined were: CAG/CAA repeat polymorphism located in the carboxyl terminal of AIB1, trinucleotide CAG repeat polymorphism in exon 1 of the AR, COMT V158M (rs4680), CYP1A1 (M1) T>C polymorphism (rs4646903) 1188bp 3'UTR, CYP1A1 (M2) I462V (rs1048943), CYP1A1 (M4) T461N (rs1799814), CYP1A2*1F C>A polymorphism in intron 1 at position -163 in the promoter (rs762551), CYP11A1 pentanucleotide repeat (TTTTA)n at position -528 in the promoter region, CYP17A1 T>C polymorphism (rs743572) located 34bp upstream from the start codon in the promoter region, CYP19A1, rs11575899 (deletion TCT) and (TTTA)n repeat polymorphism in intron 4, CYP1B1 R48G (rs10012), L432V (rs1056836) and N453S (rs1800440), estrogen receptor alpha (ESR1) GT and TA dinucleotide repeat polymorphisms located in the promoter region, GSTM1 deletion, GSTT1 deletion, GSTP1 I105V (rs1695), HSD17B1 G313S 937A>G (rs605059) and a deletion of 12bp at position -7163 in the promoter region, PGR, a G>A polymorphism at position +331 (rs10895068) and a T>C polymorphism at position -700 (rs518162), both in the promoter region and the PROGINS (V660L) polymorphism (rs1042838), SRD5A2, V89L (rs523349) and dinucleotide thymine-adenine repeat polymorphism (TA) located in the 3' untranslated region of exon 5, SULT1E1 G>A polymorphism at position -64 in promoter region (rs3736599), and UGT1A1 (A(TA)nTAA) repeat polymorphism (rs8175347).

Eight polymorphisms (COMT rs4680, CYP1A1 rs1048943, CYP1A2 rs762551, CYP1B1 rs1056836 and rs1800440, GSTP1 rs1695, HSD17β1 rs605059, and SULT1E1 rs3736599) were genotyped using the 5' nuclease assay (TaqMan®) on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers and probes were obtained from the Assay-on-Demand or Assay-by-Design service from Applied Biosystems and the PCR performed according to manufacturers' instructions. The following polymorphisms were analysed by PCR-based fragment analysis: GSTM1 deletion, GSTT1 deletion, HSD17B1 deletion 12bp and SRD5A2 rs523349. PCR-based restriction fragment length polymorphism (RFLP) analysis was used to genotype eight polymorphisms; CYP1A1 rs4646903 and rs1799814, CYP17A1 rs743572, CYP1B1 rs10012, PGR rs10895068, rs518162 and rs1042838 and SRD5A2

TA dinucleotide repeat polymorphism. The repeat polymorphisms in AIB1, AR, CYP11A1, CYP19A1, ESR1 and UGT1A1 were genotyped by the CEQ™ 8000 Genetic DNA Analysis System (Beckman Coulter, Krefeld, Germany). Genotyping conditions are available upon request.

The genotyping results were confirmed by a second laboratory research assistant and 5% of the samples were re-genotyped with 100% concordance. Any sample where a genotype could not be accurately assessed was re-genotyped. If it failed a second time, it was discarded from the analysis. The overall call rates were in the range from 90.04-100%.

Statistical Analysis

Power calculations were performed using the PS Power and Sample Size Calculations program (Dupont *et al.* 1997). The number of cases and controls were chosen to detect a 2-fold increased risk, assuming a dominant genetic model, minor allele frequency of 6.5%, p=0.05, 80% power and 1.52 control/case ratio. For each polymorphism, Hardy-Weinberg Equilibrium (HWE) was calculated in the control group to check for compliance using the Institute for Human Genetics, statistics website, http://ihg.gsf.de/ihg/polymorphisms.html (Munich, Germany). To determine differences in genotype frequencies and environmental and reproductive risk factors between the cases and controls, chi-squared (χ^2) statistics and odds ratios were calculated using unconditional logistic regression. Multivariate unconditional logistic regression was performed to determine if any risk factors altered the significance of the genotype frequency results. The risk factors taken into account were: age (continuous variable), body mass index (BMI) (<25kg/m^2 versus >=25kg/m^2), diabetes (yes/no), high blood pressure (HBP) (yes/no), hormone therapy (HT) (yes/no), personal history of cancer (yes/no), smoking (ever/never) and alcohol consumption (ever/never). T-tests were used to determine differences in the age of diagnosis of endometrial cancer and genotype. Linkage disequilibrium (LD) was tested applying Lewontin's D' statistic using the pwld function in STATA. Associations of single haplotypes and combinations of haplotypes with endometrial cancer risk were performed using SIMHAP (McCaskie *et al.* 2004). The significance levels of all tests were set at p<0.05 and were two-sided.

For the repeat polymorphisms, allele frequencies were summarised by the mean. To determine any differences in allele frequency between the cases and controls, T-tests were used. In addition, all alleles were divided into two groups based on the median of the control group. One group contained those with the short (S)

alleles (<=median) and the other with long (L) alleles (>median). As a result, three genotype groups were created: those with two short alleles (SS), one short and one long allele (SL) and two long alleles (LL). Chi-squared statistics and odds ratios were calculated for the repeat polymorphisms to determine differences in genotype frequency between the cases and controls, using unconditional logistic regression.

The genotype frequencies of all polymorphisms were compared in the case group stratified for the environmental and reproductive risk factors by using chi-squared (χ^2) analysis and ORs and 95% CIs were calculated using unconditional logistic regression. To avoid chance results due to multiple testing of the 28 polymorphisms and 16 environmental/reproductive risk factors; BMI (<25kg/m^2 versus >=25kg/m^2), age of menarche (<=12 years versus >12 years), age of menopause (<=50 years versus >50 years), HBP (yes/no), diabetes (yes/no), HT (yes/no), family history of colorectal, breast, ovarian or uterine cancer (yes/no), stage of cancer (1A to IVB), grade (1/2/3), histology (adenocarcinoma versus other), recurrence (yes/no), oral contraceptive use (yes/no) and personal history of cancer (yes/no), Bonferroni correction was used and the significance level was lowered to p=0.003 (p=0.05/16).

All statistical analysis was performed with SIMHAP (Laboratory for Genetic Epidemiology, Western Australian Institute for Medical Research, Australia) (McCaskie *et al.* 2004), Intercooled STATA 8.2 (Stata Corp., College Station, TX, USA), SPSS Version 15 (SPSS Inc. Chicago, IL, USA) and GraphPad Instat version 3.06 (GraphPad Software, San Diego, CA, USA).

Results

Comparison of selected environmental and reproductive risk factors between cases and controls

Cases and controls were different with respect to potential endometrial cancer risk factors, including HBP, diabetes, HT, alcohol consumption, personal history of any cancer, personal history of ovarian cancer and cervical cancer and history of other cancer. The characteristics of the cases and controls are shown in table 1.

Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD)

The distributions of the genotypes for all single nucleotide polymorphisms (COMT, CYP1A1, CYP1B1, CYP1A2, CYP17A1, CYP19A1, GSTP1, HSD17B1, PGR, SRD5A2 and SULT1E1) among the controls did not deviate from HWE. Polymorphisms

in CYP1A1 (rs4646903, rs1048943 and rs1799814), CYP1B1 (rs10012, rs1056836 and rs1800440), HSD17B1 (rs605059 and a 12 bp deletion), PGR (rs518162, rs10895068 and rs1042838) and SRD5A2 (rs523349 and (TA)n repeat polymorphism) were in high or complete linkage disequilibrium. D' values are shown in table 2.

Comparison of genotype and allele frequencies of repeat length polymorphisms among endometrial cancer cases and controls

The median allele length of the ESR1 (GT)n repeat polymorphism was significantly different between cases and controls, 19 and 16, respectively (T-test, p=0.02, table 3). There were no other significant associations observed for the other repeat polymorphisms. The combination of both ESR1 repeat polymorphisms, ESR1 (TA)n and ESR1 (GT)n, revealed a significant difference of median allele length between the cases and controls, 35 and 32, respectively (T-test, p=0.009, see table 3). Additionally, the genotype distribution of the repeat polymorphisms, ESR1 (GT)n and AR (CAG)n, were associated with a significant difference in endometrial cancer risk (table 4). For the ESR1 (GT)n repeat polymorphism, women with one or two long alleles had an increased risk of developing endometrial cancer in comparison to those women with two short alleles. Conversely for the AR (CAG)n repeat polymorphism, women with the combination of two short alleles or one short and one long allele had a decreased risk of developing endometrial cancer compared to women with two long alleles (table 4).

Comparison of genotype and allele frequencies of polymorphisms among endometrial cancer cases and controls

The genotype frequencies were compared between the cases and controls and significant differences were observed. The GSTM1 deletion was associated with a decreased risk of developing endometrial cancer in comparison to women without the deletion. Furthermore, the CYP1B1 rs1800440 polymorphism was also associated with a decreased risk of developing endometrial cancer since the controls had a higher frequency of the homozygous variant genotype (GG) compared to the cases (table 5).

Genotype frequencies in the cases stratified for environmental/reproductive risk factors

This analysis focused on all of the polymorphisms in the cases stratified for known environmental/reproductive confounders. After the results were adjusted using Bonferroni correction, there was only one highly significant result. Women with the ESR1 (GT)n LL genotype had a higher frequency of family members with ovarian

cancer compared to women with the SS and SL genotypes (Chi-squared p=0.002 and OR 4.667 95% CI 1.604-13.579, p=0.0059).

Influences of Genetic and Environmental/Reproductive Risk Factors on the Age of Diagnosis of Endometrial Cancer

T-tests were used to evaluate the influence of the polymorphisms on the age of diagnosis of endometrial cancer. Women carrying the CYP1A1 rs1048943 variant genotypes had an earlier median age of disease diagnosis compared to women carrying the wild-type genotype (57.8 years versus 63.6 years, respectively). The results showed that there was an age effect associated with the CYP1A1 rs1048943 polymorphism (T-test p=0.032).

Haplotype frequencies for all polymorphisms

Haplotype frequencies were estimated for the polymorphisms in CYP1A1, CYP1B1, CYP19A1, GST's, HSD17B1, PGR and SRD5A2. Significant associations were observed for CYP1A1, CYP1B1 and the GSTs. For CYP1A1, the haplotype containing the variant allele for rs1048943 and the wild-type alleles for rs4646903 and rs1799814 was significantly associated with a decreased risk of developing endometrial cancer. Similarly for CYP1B1 and the GSTs, three and two haplotypes, respectively, were associated with a decreased risk of developing endometrial cancer. Before adjustment for endometrial cancer risk factors, the CYP19A1 haplotype containing the wild type allele for the TCT deletion and the variant allele of greater than 8 repeats for the (TTTA)n repeat polymorphism were associated with a decreased risk of endometrial cancer. However, after adjustment, this association was no longer significant, see table 6.

Discussion

Endometrial cancer is considered to be a hormonally related disease. However, variations in the genes that control the production and metabolism of these hormones and their relationship with endometrial cancer have not been elucidated. Genes that control hormone biosynthesis and the metabolism of estrogen are thought to be implicated in the initiation and progression of disease. Endometrial cancer is regarded as a multifactorial disease and it is highly likely that a number of genetic differences in combination with environmental and reproductive factors alter the development of disease. The study reported herein assessed 28 polymorphisms in 18 genes involved

with hormone biosynthesis and estrogen metabolism and their association with endometrial cancer risk.

The current study took into account a series of environmental and reproductive risk factors for both the cases and controls (BMI, HBP, Diabetes, HRT, history of cancer, smoking and alcohol use). The results support previous epidemiological data for the listed risk factors indicating that there were no unusual environmental characteristics associated with the study population.

To determine if the polymorphisms were associated with an altered risk of developing endometrial cancer, we examined the relationship of each polymorphism in women with endometrial cancer compared to women without endometrial cancer. Significant associations were reported for the CYP1B1 rs1800440 polymorphism and the GSTM1 deletion. For the CYP1B1 rs1800440 polymorphism, the variant genotype (GG) compared to the combination of the wild-type and heterozygous (AA + AG) genotypes, was related to a decreased risk of developing endometrial cancer. However, these findings are preliminary due to the small number of GG carriers. In addition, the GSTM1 deletion was associated with a decreased risk of developing endometrial cancer in comparison to women with the wild-type GSTM1 genotype.

Previous association studies of polymorphisms in CYP1B1 have mainly focused on the rs1056836 polymorphism and showed inconsistent results. However, the majority of these studies suggest that there is no overall relationship with endometrial cancer (Sasaki *et al.* 2003; McGrath *et al.* 2004; Rylander-Rudqvist *et al.* 2004; Doherty *et al.* 2005; Rebbeck *et al.* 2006; Tao *et al.* 2007; Hirata *et al.* 2008). Specifically for the rs1800440 polymorphism of CYP1B1, two studies found no association with endometrial cancer risk (Sasaki *et al.* 2003; Rylander-Rudqvist *et al.* 2004), however, one study by McGrath *et al.* (2004) revealed that women with the variant genotypes (AG+GG) had a decreased risk of endometrial cancer compared to women with the wild-type (AA) genotype (McGrath *et al.* 2004). Moreover, they found no association of the CYP1B1 rs1056836 polymorphism and endometrial cancer risk (McGrath *et al.* 2004). The results reported herein for the CYP1B1 rs1056836 and rs1800440 polymorphisms are in concordance with the McGrath study however for the CYP1B1 rs1800440 polymorphism, McGrath *et al.* showed a trend for decreased risk with the combination of the variant (AG+GG) genotypes, but for this study, a significant difference was only observed for the GG genotype alone compared to the wild-type and heterozygous genotypes (AA+AG). In addition, haplotype analysis of the three

CYP1B1 polymorphisms, rs10012, rs1056836 and rs1800440, revealed three significant findings with the following genotype combinations: wild-type rs10012, rs1056836 and variant rs1800440; wild-type rs10012, rs1800440 and variant rs1056836; and wild-type rs1056836, rs1800440 and variant rs10012. These three combinations were all related to a decreased risk of endometrial cancer development and suggest that genetic variation in CYP1B1 is correlated with a decrease in endometrial cancer risk. The functional studies reported for the CYP1B1 rs10012, rs1056836 and rs1800440 polymorphisms reveal that the variant genotypes display a 2.4 to 3.4 fold higher catalytic efficiency than the wild-type genotypes. Previous studies have proposed that the higher catalytic activity of CYP1B1 may lead to higher levels of 4-hydroxyestrogens, and consequently increase cancer risk (Hanna *et al.* 2000; Aklillu *et al.* 2002). Further studies are required to examine all genetic variation in CYP1B1 in relation to catechol estrogen formation to determine the overall effect that the polymorphisms have on the activity of CYP1B1.

In this study, we found that the GSTM1 null genotype was significantly associated with a decreased risk of endometrial cancer development since the control population had a higher frequency of the null genotype compared to women with endometrial cancer. A number of studies examining endometrial cancer and the GSTM1 deletion have been reported; however, the results have been inconsistent. One study by Esteller *et al.* (1997) reported an increased risk of developing endometrial cancer with the GSTM1 deletion however they found no association with the GSTT1 deletion (Esteller *et al.* 1997). Conversely, Doherty *et al.* (2005) reported a relationship between the GSTT1 deletion and endometrial cancer risk but no significant findings were reported for the GSTM1 deletion (Doherty *et al.* 2005). In the current study, we found that the GSTM1 deletion was associated with a decrease in endometrial cancer risk since the controls had a higher frequency of the deletion in comparison to the cases. In regards to the GSTT1 and GSTP1 polymorphisms, no significant results were observed. Furthermore, haplotype analysis of all three GSTs, GSTM1, GSTT1 and GSTP1 revealed that the combination of the GSTM1 deletion, GSTT1 wild-type and GSTP1 wild-type (A) allele were related to a decrease in endometrial cancer risk. The risk was even lower in women with the haplotype containing the GSTM1 deletion, GSTT1 wild-type allele and the GSTP1 variant (G) allele. These results suggest that variation of GSTM1 and GSTP1 are associated with a decreased risk of developing endometrial cancer. The exact mechanisms of all GSTs are not clear; however it appears that GSTs deactivate metabolites of estrogen formed during phase II estrogen metabolism. The null genotypes decrease the enzymatic activity and have been

proposed to increase cancer risk due to the inability to detoxify metabolites of estrogen. However, the seven classes of cytosolic GSTs have approximately 60% homology and it is possible that other GSTs catalyse the conjugation of glutathione of a wide variety of metabolites.

Furthermore, these enzymes, CYP1B1, GSTM1 and GSTP1 are involved in the detoxification of xenobiotics and it can not be ruled out that loss of enzymatic function of CYP1B1, GSTM1 and GSTP1 is potentially a protective combination.

The analysis of the repeat polymorphisms revealed a number of significant findings. By examining the median of the repeat polymorphisms between the cases and controls, the median of the ESR1 (GT)n repeat polymorphism was significantly different since the cases had a higher repeat number than the controls, suggesting that higher repeat number is related to increased endometrial cancer risk. In addition, the two repeat polymorphisms in ESR1, (GT)n and (TA)n were combined and the results revealed an even higher increase in disease risk for women with a higher combined repeat number in comparison to those women without endometrial cancer. In addition, the repeat polymorphisms were grouped into genotypes depending on the median. Two significant results were identified: ESR1 (GT)n, women with one or two long repeats had a higher risk of developing endometrial cancer and, AR (CAG)n, women without endometrial cancer had a high frequency of the two long alleles, suggesting that longer allele length is associated with a decreased risk of developing endometrial cancer.

The functional significance of the ESR1 (GT)n and (TA)n repeat polymorphisms remains unclear. The ESR1 (GT)n repeat polymorphism has only been recently discovered and genotyped in two previous study of breast cancer patients (Cai *et al.* 2003; Boyapati *et al.* 2005). Cai *et al.* (2003) proposed that the ESR1 (GT)n repeat polymorphism may interfere with transcriptional processes as it is located in the promoter of ESR1 (Sand *et al.* 2002; Cai *et al.* 2003) and that this repeat polymorphism may affect the expression of other downstream targets by influencing transcription and/or stability of mRNA (Cai *et al.* 2003). The ESR1 (TA)n repeat polymorphism has been studied in a number of cancers, including endometrial cancer (Weiderpass *et al.* 2000). Weiderpass *et al.* (2000) reported a significant increase in risk with increasing number of short alleles (<19) for the ESR1 (TA)n repeat polymorphism. The results reported herein do not confirm this association. For ESR1 (TA)n alone, we did not find a significant association however when combined with ESR1 (GT)n, increasing number

of long alleles was related to an increase in disease risk and it is likely that this result is influenced by the significance of the ESR1 (GT)n repeat polymorphism. Therefore, the results suggest that increasing number of repeats of the ESR1 (GT)n repeat polymorphism is associated with an increase in endometrial cancer risk.

Specifically for the AR (CAG)n repeat polymorphism, longer repeat length (>22 repeats) has been correlated with decreased receptor activity (Chamberlain *et al.* 1994; Irvine *et al.* 2000; Buchanan *et al.* 2004) and consequently may decrease the ability to synthesise androgens and to recruit co-regulators that are capable of increasing transcription of androgen-related genes, such as the estrogen receptors. Four previous studies of the (CAG)n repeat polymorphism in the AR and risk of endometrial cancer have yielded inconsistent results; two small epidemiological studies reported a positive association (Yaron *et al.* 2001; Sasaki *et al.* 2003), one study observed no significant findings (Ju *et al.* 2007) and another showed that higher repeat length was associated with decreased risk (McGrath *et al.* 2006). The results of this study are in concordance with the latter study. The study reported herein and the one of McGrath *et al.* (2006) had a much larger sample population than the other three studies (Yaron *et al.* 2001; Sasaki *et al.* 2003; Ju *et al.* 2007), decreasing the possibility of type I and II statistical errors. These results suggest that the high frequency of women without endometrial cancer who have a longer number of the AR (CAG)n repeats are protected from endometrial cancer development since longer repeat length decreases hormone levels thereby affecting the risk of developing endometrial cancer.

We have also shown using haplotype analysis that the CYP1A1 haplotype containing the wild-type alleles for rs4646903 and rs1799814 and the variant allele of rs1048943 was associated with a decreased risk of developing endometrial cancer. The minor allele frequency in the cases is very small and for that reason this result should be interpreted with caution. Doherty *et al.* (2005) examined polymorphisms in the major genes involved in estrogen metabolism and found that individuals that carried at least one CYP1A1 rs4646903 or rs1048943 variant allele had a decreased risk of developing endometrial cancer (Doherty *et al.* 2005). In addition, combined analysis of CYP1A1, CYP1A2 and CYP1B1 revealed that women with a low-risk haplotype, based on the functional analysis of these polymorphisms, had a reduced risk of developing endometrial cancer (Doherty *et al.* 2005). Conversely, another study by Rebbeck *et al.* (2006) examined a number of polymorphisms involved in estrogen biosynthesis and metabolism and found that the rs1048943 polymorphism in CYP1A1 was associated with an increased risk of developing endometrial cancer. The results of this study are in

concordance with the study by Doherty *et al.* (2005) and functional data that suggests that the CYP1A1 M2 rs1048943 polymorphism increase enzymatic activity (Petersen *et al.* 1991), thereby increasing production of 2-hydroxyestrogens. Taken together, these results must be studied in a larger independent data set to verify the association of CYP1A1 variants and endometrial cancer risk.

Stratification of the case group for each of the environmental/reproductive risk factors revealed only one significant result after adjustment for multiple testing using the Bonferroni correction. Cases with a family history of ovarian cancer had a higher frequency of long repeat allele length of the ESR1 (GT)n repeat polymorphism in comparison to cases without a family history of ovarian cancer. This repeat polymorphism has not previously been examined in ovarian cancer and therefore these results warrant further investigation to explore the possible association of the ESR1 (GT)n repeat polymorphism and ovarian cancer risk.

For all of the other polymorphisms examined in this study, there were no significant associations found comparing genotype and haplotype frequencies between the cases and controls and examining the age of diagnosis of endometrial cancer. It is possible that these SNPs do not alter individual susceptibility to endometrial cancer although further studies harbouring larger sample populations are required to confirm this association.

There have been a very limited number of studies that have examined genetic variation in the major pathways of hormone biosynthesis and estrogen metabolism and endometrial cancer risk. The main risk factor for developing disease is exposure to unopposed or excessive estrogen and for this reason, knowledge about the genes involved in the biosynthesis and metabolism of estrogen and the genetic variation that occurs within these genes, is an essential step for the identification of risk factors associated with disease.

A number of strengths of this study include our cohort of matched cases and controls from the same geographical region, the replication of results thereby eliminating genotyping errors and a sufficient cohort size to detect significant associations with adequate power. There are, however, a number of limitations. With this cohort, haplotype analysis of multiple polymorphisms in the same genes was able to be performed but a larger cohort is required to examine the combinations of different genes involved in the same pathways to determine differences in risk of endometrial

cancer. The adjustment of specific endometrial cancer risk factors varies from study to study and variation in risk factor information obtained could possibly be implicated in the differences observed between different populations. In this study, the analysis took into account the major sources of risk which included: age, BMI, HT, history of cancer, smoking, alcohol use, HBP and diabetes. Confirmation of the results obtained in this study must be performed by an independent and larger cohort.

In conclusion, we have reported that polymorphisms in the AR, CYP1A1, CYP1B1, ESR1, GSTM1 and GSTP1 are associated with altered risk of endometrial cancer development. These polymorphisms may be new biomarkers for genetic susceptibility to endometrial cancer.

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Table 1: Comparison of Environmental and Reproductive Risk Factors between Cases and Controls.

Risk Factor	Group	Cases n (%)	Controls n (%)	OR	95% CI	P value
BMI (<25kg/m^2 and >=25kg/m^2)[A]	<25kg/m^2	34 (19.1)	72 (24.7)	0.718	0.454-1.136	p=0.157
	>=25kg/m^2	144 (80.9)	219 (75.3)			
High Blood Pressure (yes/no)	yes	107 (56.0)	114 (39.2)	1.978	1.366 – 2.864	p<0.001
	no	84 (44.0)	177 (60.8)			
Diabetes (yes/no)	yes	44 (23.0)	31 (10.7)	2.51	1.519 – 4.148	p<0.001
	no	147 (77.0)	260 (89.3)			
Hormone Therapy (yes/no)	yes	47 (24.6)	40 (13.7)	2.048	1.282 – 3.273	p=0.003
	no	144 (75.4)	251 (86.3)			
Smoking (ever/never)	ever	52 (27.2)	68 (23.4)	1.227	0.807 – 1.865	p=0.338
	never	139 (72.8)	223 (76.6)			
Alcohol consumption (ever/never)	ever	92 (48.2)	228 (78.4)	0.257	0.172 – 0.382	p<0.001
	never	99 (51.8)	63 (21.6)			
Personal History of Any Cancer (yes/no)	yes	51 (26.7)	28 (9.6)	3.422	2.066 – 5.667	p<0.001
	no	140 (73.3)	263 (90.4)			
History of Ovarian or Cervical Cancer (yes/no)	yes	15 (7.9)	3 (1.0)	8.182	2.335 – 28.663	p=0.001
	no	176 (92.1)	288 (99.0)			
Ovarian Cancer (yes/no)	yes	7 (3.7)	1 (0.3)	11.033	1.346 – 90.403	p=0.025
	no	184 (96.3)	290 (99.7)			
Cervical Cancer (yes/no)	yes	8 (4.2)	2 (0.7)	6.317	1.327 – 30.077	p=0.021
	no	183 (95.8)	289 (99.3)			
History of Skin Cancer (yes/no)	yes	20 (10.5)	19 (6.5)	1.674	0.869 – 3.228	p=0.124
	no	171 (89.5)	272 (93.5)			
History of Bowel Cancer (yes/no)	yes	10 (5.2)	8 (2.7)	1.954	0.757 – 5.045	p=0.166
	no	181 (94.8)	283 (97.3)			
History of Other Cancer (yes/no)	yes	10 (5.2)	4 (1.4)	3.964	1.255 – 12.828	p=0.022
	no	181 (94.8)	287 (98.6)			

BMI, body mass index.

Note: BMI not known for 13 cases.

Table 2: Lewontin's D' statistic linkage disequilibrium results for CYP1A1, CYP1B1, HSD17B1, PGR and SRD5A2 polymorphisms

Gene	Polymorphisms	D' values
CYP1A1	rs4646903 + rs1048943	1.00
	rs4646903 + rs1799814	0.90
	rs1048943 + rs1799814	0.01
CYP1B1	rs10012 + rs1056836	0.99
	rs10012 + rs1800440	0.78
	rs1056836 + rs1800440	1.00
HSD17B1	rs605059 and 12bp deletion	1.00
PGR	rs518162 + rs10895068	0.63
	rs518162 + rs1042838	0.85
	rs10895068 + rs1052838	0.91
SRD5A2	rs523349 + 0/9 TA repeat polymorphism	1.00

Table 3: Medians of the repeat lengths of AIB1 (CAG/CAA)n, AR (CAG)n, CYP11A1 (TTTTA)n, CYP19A1 (TTTA)n, ESR1 (TA)n and (GT)n, UGT1A1 (TA)n, and ESR1 (TA)n and (GT)n combined for cases and controls

Repeat Polymorphism	Median	Range of no. of repeats	p-value
AIB1 (CAG/CAA)n			
Cases	28.5	26 - 30	0.7
Controls	28	26 - 29	
AR (CAG)n			
Cases	22	11 - 33	0.87
Controls	22	13 - 37	
CYP11A1 (TTTTA)n			
Cases	4	4 - 9	0.45
Controls	4	4 - 9	
CYP19A1 (TTTA)n			
Cases	8	7 - 13	0.7
Controls	8	7 - 13	
ESR1 (TA)n			
Cases	16	10 - 34	0.15
Controls	16	10 - 27	
ESR1 (GT)n			
Cases	19	13 - 23	**0.02**
Controls	16	13 - 23	
UGT1A1 (TA)n			
Cases	6	5 - 7	0.59
Controls	6	5 - 8	
ESR1 (GT)n and ESR1 (TA)n combined			
Cases	35	25 - 43	**0.009**
Controls	32	25 - 41	

Table 4: ESR1 (TA)n and AR (CAG)n repeat polymorphisms, divided into short (S) and long (R) repeat allele length, and endometrial cancer risk

Polymorphism	Genotype	Cases n (%)	Controls n (%)	χ^2	OR (95% CI) and p value	
ESR1 (GT)n	SS	39 (20.9)	86 (30.6)	p=0.059	1.00 (reference)	
	SL	93 (49.7)	127 (45.2)		**1.909 (1.118-3.261)$_{adj\#}$**	**p=0.018**
					1.615 (1.016-2.567)	**p=0.043**
	LL	55 (29.4)	68 (24.2)		**1.841 (1.009-3.359)$_{adj\#}$**	**p=0.047**
					1.784 (1.061-2.997)	**p=0.029**
	SS+SL	132 (70.6)	213 (75.8)	p=0.210†	1.215 (0.748-1.973)$_{adj\#}$	p=0.432
					1.305 (0.861-1.979)	p=0.210
	SL+LL	148 (79.1)	195 (69.4)	**p=0.02‡**	**1.889 (1.145-3.117)$_{adj\#}$**	**p=0.013**
					1.674 (1.084-2.585)	**p=0.020**
AR (CAG)n	SS	66 (35.9)	97 (39.0)	p=0.033	1.00 (reference)	
	SL	94 (51.1)	100 (40.2)		1.486 (0.914-2.418)$_{adj\#}$	p=0.110
					1.382 (0.907-2.104)	p=0.132
	LL	24 (13.0)	52 (20.9)		0.605 (0.311-1.178)$_{adj\#}$	p=0.140
					0.678 (0.381-1.207)	p=0.187
	SS+SL	160 (87.0)	197 (79.1)	**p=0.034†**	**0.484 (0.263-0.892)$_{adj\#}$**	**p=0.020**
					0.568 (0.336-0.962)	**p=0.035**
	SL+LL	118 (64.1)	152 (61.0)	p=0.512‡	1.168 (0.741-1.839)$_{adj\#}$	p=0.504
					1.141 (0.769-1.693)	p=0.512

SS = median values and values below the median, SL = one allele; median values and values below the median, other allele; values greater than the median, LL = both alleles with values greater than the median.

Odds Ratio adjusted for age, BMI, diabetes, HBP, history of cancer, HT, smoking and alcohol use.

† The combination of two short alleles (SS) and one short and one long allele (SL) versus both long alleles (LL); SS+SL versus LL.

‡ Two short alleles (SS) versus the combination of one short and one long allele (SL) in combination with two long alleles (LL); SS versus SS+SL.

Table 5 (part 1): Genotype frequencies of polymorphisms involved in hormone biosynthesis and estrogen metabolism and endometrial cancer risk

Polymorphism	Genotypes	Cases n (%)	Controls n (%)	χ2	OR (95% CI) and p value
COMT rs4680	GG	38 (19.9)	64 (22.1)	p=0.791	1.256 (0.742-2.126) p=0.397
	GA	98 (51.3)	149 (51.4)		
	AA	55 (28.8)	77 (26.6)		
	GA+AA	153 (80.1)	226 (77.9)		
CYP1A1 rs4646903	TT	162 (84.8)	238 (82.9)	p=0.136	0.879 (0.493-1.566) p=0.661
	TC	23 (12.0)	46 (16.0)		
	CC	6 (3.1)	3 (1.0)		
	TC+CC	29 (15.2)	49 (17.1)		
CYP1A1 rs1048943	AA	177 (92.7)	257 (94.8)	p=0.208	1.234 (0.486-3.131) p=0.658
	AG	12 (6.3)	14 (5.2)		
	GG	2 (1.0)	0 (0.0)		
	AG+GG	14 (7.3)	14 (5.2)		
CYP1A1 rs1799814	AA	182 (95.3)	268 (92.4)	p=0.209	0.560 (0.221-1.415) p=0.220
	AG	9 (4.7)	0 (0.0)		
	GG	0 (0.0)	0 (0.0)		
	AG+GG	9 (4.7)	22 (7.6)		
CYP1A2 rs762551	CC	107 (56.0)	161 (55.3)	p=0.820	1.102 (0.724-1.675) p=0.651
	CA	73 (38.2)	109 (37.5)		
	AA	11 (5.8)	21 (7.2)		
	CA+AA	84 (44.0)	130 (44.7)		
CYP1B1 rs10012	CC	91 (47.6)	130 (47.1)	p=0.390	1.102 (0.718-1.690) p=0.656
	CG	88 (46.1)	119 (43.1)		
	GG	12 (6.3)	27 (9.8)		
	CG+GG	100 (52.4)	146 (52.9)		
CYP1B1 rs1056836	GG	71 (37.2)	101 (34.8)	p=0.870	0.861 (0.558-1.331) p=0.502
	GC	88 (46.1)	139 (47.9)		
	CC	32 (16.8)	50 (17.2)		
	GC+CC	120 (62.8)	189 (65.2)		
CYP1B1 rs1800440	AA	132 (69.1)	183 (63.5)	p=0.078	
	AG	57 (29.8)	92 (31.9)		
	GG	2 (1.0)	13 (4.5)		
	AG+GG	59 (30.9)	105 (36.5)		0.693 (0.442-1.088) p=0.111
	AA+AG	189 (99.0)	275 (95.5)		**0.108 (0.013-0.898) p=0.039***
CYP17 rs743572	TT	73 (38.4)	113 (40.4)	p=0.914	0.869 (0.560-1.348) p=0.531
	TC	88 (46.3)	126 (45.0)		
	CC	29 (15.3)	41 (14.6)		
	TC+CC	117 (61.6)	167 (59.6)		
CYP19 rs11575899	No deletion	102 (53.7)	122 (46.4)	p=0.157	0.775 (0.506-1.187) p=0.242
	Deletion	88 (46.3)	141 (53.6)		
GSTM1 deletion	No deletion	107 (56.0)	125 (43.7)	**p=0.008**	**0.564 (0.365-0.873) p=0.010**
	Deletion	84 (44.0)	161 (56.3)		
GSTT1 deletion	No deletion	158 (82.7)	239 (83.6)	p=0.809	1.109 (0.635-1.934) p=0.716
	Deletion	33 (17.3)	47 (16.4)		

X^2 – Wild Type Genotype versus Heterozygous genotype versus Homozygous Variant genotype.

Odds Ratios – Wild Type genotype compared to combination of heterozygous and homozygous variant genotypes. All Odds Ratios were adjusted for age, BMI, diabetes, HBP, history of cancer, HT, smoking and alcohol use.

* CYP1B1 rs1800440 – Combined genotypes AA and AG compared to GG.

Table 5 (part 2): Genotype frequencies of polymorphisms involved in hormone biosynthesis and estrogen metabolism and endometrial cancer risk

Polymorphism	Genotypes	Cases n (%)	Controls n (%)	χ2	OR (95% CI) and p value
GSTP1 rs1695	AA	87 (45.5)	110 (37.9)	p=0.085	0.720 (0.470-1.102) p=0.130
	AG	88 (46.1)	139 (47.9)		
	GG	16 (8.4)	41 (14.1)		
	AG+GG	104 (54.5)	180 (62.1)		
HSD17B1 -7163 del12	No deletion	105 (55.0)	157 (55.9)	p=0.833	1.075 (0.699-1.051) p=0.743
	Deletion	86 (45.0)	124 (44.1)		
HSD17B1 rs605059	AA	64 (33.5)	91 (31.4)	p=0.291	0.973 (0.617-1.532) p=0.905
	AG	84 (44.0)	147 (50.7)		
	GG	43 (22.5)	52 (17.9)		
	AG+GG	127 (66.5)	199 (68.6)		
PGR rs518162	GG	167 (87.4)	250 (88.7)	0.693	1.410 (0.749-2.654) p=0.288
	GA	23 (12.0)	29 (10.3)		
	AA	1 (0.5)	3 (1.1)		
	GA+AA	24 (12.6)	32 (11.3)		
PGR rs10895068	GG	163 (85.3)	249 (86.2)	p=0.967	1.087 (0.591-2.000) p=0.788
	GA	26 (13.6)	37 (12.8)		
	AA	2 (1.0)	3 (1.0)		
	GA+AA	28 (14.7)	40 (13.8)		
PGR rs1042838	GG	128 (67.0)	197 (68.6)	p=0.840	0.884 (0.561-1.392) p=0.595
	GT	56 (29.3)	82 (28.6)		
	TT	7 (3.7)	8 (2.8)		
	GT+TT	63 (33.0)	90 (31.4)		
SRD5A2 rs523349	GG	87 (45.5)	156 (54.0)	p=0.187	1.428 (0.934-2.184) p=0.100
	GC	89 (46.6)	112 (38.8)		
	CC	15 (7.9)	21 (7.3)		
	GC+CC	104 (54.5)	133 (46.0)		
SRD5A2 (AT)n	0	156 (81.7)	222 (76.3)	p=0.293	0.747 (0.442-1.263) p=0.276
	0/9	35 (18.3)	68 (23.4)		
	9/9	0 (0.0)	1 (0.3)		
	0/9+9/9	35 (18.3)	69 (23.7)		
SULT1E1 rs3736599	GG	159 (83.2)	244 (83.8)	p=0.797	1.181 (0.674-2.068) p=0.562
	GA	31 (16.2)	44 (15.1)		
	AA	1 (0.5)	3 (1.0)		
	GA+AA	32 (16.8)	47 (16.2)		

X^2 – Wild Type Genotype versus Heterozygous genotype versus Homozygous Variant genotype.

Odds Ratios – Wild Type genotype compared to combination of heterozygous and homozygous variant genotypes. All Odds Ratios were adjusted for age, BMI, diabetes, HBP, history of cancer, HT, smoking and alcohol use.

Table 6: Haplotype Analysis of CYP1A1, CYP1B1, CYP19A1 and GSTs and endometrial cancer risk

Gene	Haplotype	Frequency of Cases in % (SE)	Frequency of Controls in % (SE)	OR (95% CI)	p value
CYP1A1 rs4646903, rs1048943 and rs1799814	**TAC**	88.66 (0.016)	79.25 (0.017)	1.00 (reference)	
	CAC	5.24 (0.011)	8.04 (0.011)	0.907 (0.482-1.700)$_{adj^}$	p=0.728
				0.676 (0.403-1.133)	p=0.135
	TGC	0.27 (0.003)	6.57 (0.010)	**0.157 (0.273-0.811)$_{adj^}$**	**p=0.015**
				0.172 (0.038-0.738)	**p=0.0098**
	TAA	1.91 (0.007)	3.19 (0.007)	0.450 (0.138-1.474)$_{adj^}$	p=0.197
				0.545 (0.230-1.296)	p=0.169
	CGC	3.47 (0.009)	2.02 (0.006)	1.969 (0.497-7.197)$_{adj^}$	p=0.339
				1.595 (0.708-3.561)	p=0.261
CYP1B1 rs10012, rs1056836 and rs1800440	**CGA**	14.92 (0.018)	7.45 (0.011)	1.00 (reference)	
	CCA	39.79 (0.025)	38.42 (0.020)	**0.479 (0.268-0.858)$_{adj^}$**	**p=0.013**
				0.519 (0.333-0.809)	**p=0.0038**
	GGA	29.32 (0.023)	31.24 (0.019)	**0.470 (0.256-0.864)$_{adj^}$**	**p=0.015**
				0.507 (0.318-0.809)	**p=0.0043**
	CGG	15.97 (0.019)	18.85 (0.016)	**0.362 (0.188-0.695)$_{adj^}$**	**p=0.0023**
				0.433 (0.262-0.719)	**p=0.0012**
GSTM1 deletion, GSTT1 deletion, and GSTP1 rs1695	**No del/No del/A**	46.80 (0.026)	38.63 (0.02)	1.00 (reference)	
	No del/No del/G	25.29 (0.022)	26.30 (0.018)	0.667 (0.409-1.077)$_{adj^}$	p=0.094
				0.782 (0.547-1.114)	p=0.165
	Del/no del/A	15.03 (0.018)	17.76 (0.016)	**0.485 (0.262-0.884)$_{adj^}$**	**p=0.016**
				0.572 (0.366-0.890)	**p=0.013**
	Del/no del/G	4.24 (0.01)	7.52 (0.011)	**0.268 (0.082-0.799)$_{adj^}$**	**p=0.011**
				0.402 (0.182-0.859)	**p=0.016**
	No del/del/A	4.04 (0.01)	4.18 (0.008)	0.671 (0.236-1.799)$_{adj^}$	p=0.397
				0.780 (0.371-1.609)	p=0.499
	Del/Del/A	2.72 (0.008)	1.12 (0.004)	1.050 (0.262-3.861)$_{adj^}$	p=0.695
				1.793 (0.509-5.830)	p=0.375
	No del/del/G	1.88 (0.007)	1.52 (0.005)	1.132 (0.215-4.891)$_{adj^}$	p=0.608
				1.019 (0.270-3.382)	p=0.643
CYP19 rs11575899 and (TTTTA)n	**No del/<=8 repeats**	15.18 (0.018)	10.14 (0.013)	1.00 (reference)	
	No del/>8 repeats	29.84 (0.023)	42.27 (0.021)	0.591 (0.322-1.054)$_{adj^}$	p=0.065
				0.595 (0.399-0.881)	**p=0.0091**
	Del/<=8 repeats	54.97 (0.026)	47.59 (0.021)	0.898 (0.500-1.566)$_{adj^}$	p=0.509
				0.867 (0.587-1.271)	p=0.436

Note: This table only contains haplotypes with a frequency greater than 1%.

Variant/deleted alleles are underlined.

^ Odds Ratio adjusted for age, BMI, diabetes, HBP, history of cancer, HT, smoking and alcohol use.

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Chapter IV

Estrogen receptor polymorphisms and the risk of endometrial cancer.

Estrogen receptor polymorphisms and the risk of endometrial cancer.

Ashton KA, Proietto A, Otton G, Symonds I, McEvoy M, Attia J, Gilbert M, Hamann U, Scott RJ. ***BJOG*** **116(8): 1053-1061**

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Abstract

OBJECTIVE: There is evidence that estrogens and some of their metabolites are involved in endometrial cancer pathogenesis. As estrogens mediate their effects via the estrogen receptors, ESR1 and ESR2, the objective of this investigation was to determine whether six single nucleotide polymorphisms (SNPs) in these two genes were over-represented in a population of endometrial cancer patients compared with a healthy matched control population, thereby associating differences in these genes with endometrial cancer.

DESIGN: The study is a case-control investigation large enough to detect a two-fold increased risk, assuming a dominant genetic model, with P = 0.05 and 80% power. SETTING: The study and control populations were all from the Hunter-New England region of New South Wales, Australia collected between the years 1992 and 2005.

POPULATION: The study consisted of 191 endometrial cancer patients and 291 healthy controls matched for gender and age. METHODS: Two SNPs in ESR1 and four SNPs in ESR2 were genotyped using PCR-based restriction fragment length polymorphism analysis and real-time PCR. Odds ratios were calculated using unconditional logistic regression and SIMHAP was used for haplotype analysis, adjusting for potential endometrial cancer risk factors. Kaplan-Meier survival analysis, Cox regression and t tests were used to examine the patient's age of diagnosis of endometrial cancer and genotype.

MAIN OUTCOME MEASURES: Over-representation of ESR1 and ESR2 polymorphisms in the endometrial cancer population compared with the control population indicates an involvement in the development and/or progression of disease. RESULTS: Two ESR1 (rs2234693 and rs9340799) and two ESR2 (rs1255998 and rs944050) polymorphisms were associated with an increased risk of endometrial cancer. Following adjustment for risk factors, the association with the ESR1 and ESR2 polymorphisms (rs2234693, rs1255998 and rs944050) remained highly significant. Haplotype analysis revealed that carriers of the ESR1 haplotype (variant alleles; rs2234693 and rs9340799) and the ESR2 haplotype (variant allele; rs1255998 and wild-type alleles; rs944050, rs4986938 and rs1256049) were at an increased risk (OR 1.862, P = 0.013 and OR 1.918, P = 0.046 respectively). This risk was even greater in women carrying both risk haplotypes (OR 5.041, P = 0.007).

CONCLUSIONS: Our data suggest that the ESR1 (rs2234693 and rs9340799) and the ESR2 (rs1255998 and rs944050) polymorphisms may be associated with an increased risk of developing endometrial cancer.

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Chapter V

Polymorphisms in TP53 and MDM2 combined are associated with high grade endometrial cancer

Polymorphisms in TP53 and MDM2 combined are associated with high grade endometrial cancer.

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Abstract

OBJECTIVES: Determinants of endometrial cancer grade have not been precisely defined, however, cell cycle control is considered to be integrally involved in endometrial cancer development. TP53 and MDM2 are essential components for cell cycle arrest and apoptosis. Polymorphisms in these genes cause TP53 inactivation and MDM2 over-expression, leading to accumulation of genetic errors.

METHODS: One polymorphism in MDM2, rs2279744 (SNP309) and three polymorphisms in TP53 rs1042522 (R72P), rs17878362 and rs1625895 were genotyped in 191 endometrial cancer cases and 291 controls using PCR-based fragment analysis, RFLP analysis and real-time PCR.

RESULTS: The results showed no associations of the three TP53 polymorphisms and MDM2 SNP309 alone or in combination with endometrial cancer risk. However, the combination of MDM2 SNP309 and the three TP53 polymorphisms was significantly associated with a higher grade of endometrial cancer (wild-type genotypes versus variant genotypes: OR 4.15, 95% CI 1.82-9.46, p=0.0003). Analysis of family history of breast cancer revealed that the variant genotypes of the three TP53 polymorphisms were significantly related to a higher frequency of family members with breast cancer in comparison to endometrial cancer cases without a family history of breast cancer (wild-type genotypes versus variant genotypes: OR 2.78, 95% CI 1.36-5.67, p=0.004).

CONCLUSIONS: The combination of the MDM2 SNP309 and the three TP53 polymorphisms appear to be related to a higher grade of endometrial cancer. The association of the endometrial cancer cases with family history of breast cancer and the three TP53 polymorphisms suggests that this constellation of malignancies may represent a low-risk familial cancer grouping.

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Chapter VI

The influence of the Cyclin D1 870 G>A polymorphism as an endometrial cancer risk factor

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The influence of the Cyclin D1 870 G>A polymorphism as an endometrial cancer risk factor

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Abstract

Background: Cyclin D1 is integral for the G1 to S phase of the cell cycle as it regulates cellular proliferation. A polymorphism in cyclin D1, 870 G>A, causes overexpression and supports uncontrollable cellular growth. This polymorphism has been associated with an increased risk of developing many cancers, including endometrial cancer.

Methods: The 870 G>A polymorphisms (rs605965) in the cyclin D1 gene was genotyped in an Australian endometrial cancer case-control population including 191 cases and 291 controls using real-time PCR analysis. Genotype analysis was performed using chi-squared (χ^2) statistics and odds ratios were calculated using unconditional logistic regression, adjusting for potential endometrial cancer risk factors.

Results: Women homozygous for the variant cyclin D1 870 AA genotype showed a trend for an increased risk of developing endometrial cancer compared to those with the wild-type GG genotype, however this result was not statistically significant (OR 1.692 95% CI (0.939–3.049), p = 0.080). Moreover, the 870 G>A polymorphism was significantly associated with family history of colorectal cancer. Endometrial cancer patients with the homozygous variant AA genotype had a higher frequency of family members with colorectal cancer in comparison to endometrial cancer patients with the GG and combination of GG and GA genotypes (GG versus AA; OR 2.951, 95% CI (1.026–8.491), p = 0.045, and GG+GA versus AA; OR 2.265, 95% CI (1.048–4.894), p = 0.038, respectively).

Conclusion: These results suggest that the cyclin D1 870 G>A polymorphism is possibly involved in the development of endometrial cancer. A more complex relationship was observed between this polymorphism and familial colorectal cancer.

Background

Cyclin D1 (CCND1) is a key protein in the regulation of the cell cycle at the G1 to S phase transition, and is essential for regulation of proliferation, differentiation and transcriptional control [1]. Overexpression of cyclin D1 induces excessive cellular proliferation and is a feature of a number of cancers, including endometrial and colorectal cancer [2-6]. Specifically for endometrial cancer, numerous studies have reported increased cellular proliferation co-existing with progressive derailment of cyclin D1, leading to the progression of hyperplasia to endometrial endometriod carcinoma [7-9]. Many association studies have focused their attention to the functionally significant 870 G>A polymorphism in cyclin D1 which creates two different splice variant transcripts [10]. The normal transcript encodes exon 5 which is essential for ubiquitin-mediated proteolysis whereas the other transcript lacks the destruction box in exon 5 and increases the half life of cyclin D1 [10]. The A allele of the 870 G>A polymorphism in cyclin D1 encodes the alternate transcript and increased levels of cyclin D1 are also evident in the heterozygous state [10,11].

Previous studies have reported inconsistent findings for the cyclin D1 polymorphism and a number of different cancers. With respect to endometrial cancer, there has been one published report on the association between the cyclin D1 870 G>A polymorphism and endometrial cancer risk in Korean women [12]. Kang et al. (2005) [12] reported that endometrial cancer patients with the AA genotype had an increased risk of disease compared to carriers of the GG genotype and the combination of the GG and GA genotypes, suggestive of a recessive model for the A allele.

Endometrial cancer is the most common gynaecological malignancy in Western countries and it is important to determine the genetic variants associated with disease since the genetic basis is poorly understood. Estrogen and its metabolites have been associated with an increased risk of developing endometrial cancer due to their ability to cause DNA damaging events [13], therefore cell cycle control is integral for the recognition, repair and/or elimination of DNA damage to prevent the initiation of cancer.

The focus of this study was to examine the 870 G>A polymorphism in cyclin D1 and its association with endometrial cancer risk in Caucasians including 191 endometrial cancer cases and 291 controls.

Methods

Study Population

This study initially consisted of 213 consecutively recruited women with histologically confirmed endometrial cancer who presented for treatment at the Hunter Centre for Gynaecological Cancer, John Hunter Hospital, Newcastle, New South Wales, Australia between the years 1992 and 2005. Women that had additionally been diagnosed with breast cancer were excluded from this study.

The final analysis included 191 endometrial cancer patients. Data on reproductive and environmental risk factors including ethnicity, body mass index (BMI), diabetes, high blood pressure (HBP), age of diagnosis of endometrial cancer, age of menarche, age of menopause, other personal cancer history, family history of cancer (defined as cancer in the index patient plus one or more first or second degree relatives diagnosed with cancer), parity, breastfeeding, oral contraceptive use, chemotherapy, radiotherapy, hormone therapy (HT), smoking and alcohol use was collected using self reported questionnaires. Information regarding recurrence, stage, grade and histology of endometrial cancer was collected from the medical records.

The control population consisted of 291 women who were recruited between the years 2004 and 2005 for the Hunter Community Study. This study aims to identify genetic and environmental factors associated with ageing in a cohort of individuals obtained from the Hunter region, Newcastle, New South Wales, Australia. Any control that had a prior diagnosis of either breast or endometrial cancer was excluded from the study. Controls were matched to cases by sex and age.

All participants provided informed written consent prior to participation in this study. Ethics approval was obtained from the Human Research Ethics Committee, University of Newcastle and the Hunter Area Research Ethics Committee, Hunter New England Health Service, Newcastle, New South Wales, Australia.

DNA Isolation

Genomic DNA was extracted from 10 ml EDTA blood as previously described [14].

Molecular Analysis

Genotyping of the cyclin D1 870 G>A polymorphism (rs603965) was performed on an ABI PRISM® 7500 Real-Time PCR System (PE Applied Biosystems, Foster City, CA), using primers and probes from Assay-by-Demand (Applied Biosystems) (assay ID: C_744725_1). The assay was performed under universal conditions previously described [15]. The genotyping results were confirmed by a second laboratory research assistant and 5% of the samples were re-genotyped with 100% concordance. Any sample where a genotype could not be accurately assessed was re-genotyped. If it failed a second time, it was discarded from the analysis. The overall call rates were in the range from 99.7–100%.

Statistical Analysis

Power calculations were performed using Quanto (Version 1.2.3, May 2007, http://hydra.usc.edu/GxE. The number of cases and controls were chosen to detect a 2-fold increased risk, assuming a dominant genetic model, minor allele frequency of 6.5%, p = 0.05, 80% power and 1.52 control/case ratio. For each polymorphism, Hardy-Weinberg Equilibrium (HWE) was calculated in the control groups to check for compliance using the Institute for Human Genetics, statistics website, http://ihg2.helmholtz-muenchen.de/ihg/snps.html (Munich, Germany). To determine differences in genotype frequencies and environmental and reproductive risk factors between the cases and controls, chi-squared (χ^2) statistics, odds ratios (ORs) and 95% confidence intervals (CI) were calculated using unconditional logistic regression. Multivariate unconditional logistic regression was performed to determine if any risk factors altered the significance of the genotype frequency results. The risk factors taken into account were: BMI (<25 and ≥ 25 kg/m²) diabetes (yes/no), HBP (yes/no), HT (yes/no), personal history of cancer (yes/no), smoking (ever/never) and alcohol consumption (ever/never). Other risk factors such as age of menopause were not included in the analysis since this information was not available for the controls.

The genotype frequencies of the cyclin D1 870 G>A polymorphism was compared in the case group stratified for the following environmental and reproductive risk factors; BMI (<25 versus >= 25), age of menarche (<12 v >= 12), age of menopause (<50 versus >= 50), parity (yes/no), oral contraceptive use (ever/never), HBP (yes/no), diabetes (yes/no), personal history of ovarian, colorectal, and/or cervical cancer (yes/no), radiotherapy (yes/no), chemotherapy (yes/no), hormone therapy (yes/no), family history of uterine, breast, colorectal and/or ovarian cancer (yes/no, defined as one first or second degree relative with cancer), smoking (ever/never), alcohol (ever/never), stage of cancer, grade of cancer, histology and cancer recurrence. This analysis was performed by using chi-squared (χ^2) analysis and ORs and 95% CIs were calculated using unconditional logistic regression.

T-tests were used to determine differences in the age of diagnosis of endometrial cancer by genotype. Kaplan Meier survival analysis was used to plot the cumulative survival versus the patient's age of diagnosis of endometrial cancer. By comparing the Kaplan-Meier survival curves for each genotype, we tested if there were differences in the age of diagnosis of endometrial cancer by genotype. The Wilcoxon's test was used to determine the significance of observations from early ages of diagnosis, log-rank test, which gives more weight to later ages and Tarone-Ware test, which is an intermediate of the two other tests were used to examine the homogeneity of the survival curves. The polymorphisms that showed a statistically significant difference between the genotypes and the age of diagnosis of endometrial cancer for all three statistical tests were further examined by a multivariate Cox regression model where a number of specific risk factors were incorporated into the analysis.

The significance levels of all tests were set at $p < 0.05$ and were two-sided. All statistical analysis was performed with Intercooled STATA 8.2 (Stata Corp., College Station, TX, USA), SPSS Version 15 (SPSS Inc. Chicago, IL, USA) and GraphPad Instat version 3.06 (GraphPad Software, San Diego, CA, USA).

Results

Cases and controls were different with respect to potential endometrial cancer risk factors, including HBP, diabetes, HT, alcohol consumption, personal history of any cancer, personal history of ovarian cancer, cervical cancer and other cancers. The characteristics of the cases and controls are shown in table 1. The distributions of the cyclin D1 genotypes among the controls did not deviate from HWE.

The genotype frequencies were compared between the cases and controls for the cyclin D1 870 G>A polymorphism, however no significant differences were observed (table 2). However, there was a trend for increased risk of developing endometrial cancer for women with the AA genotype compared to those with the GG genotype (OR_{adj} 1.692 95% CI (0.939–3.049), p = 0.080).

Kaplan-Meier survival analysis and T-tests were used to evaluate the influence of the cyclin D1 870 G>A polymorphism on the age of diagnosis of endometrial cancer. No significant differences were observed (data not shown).

Family histories of other cancers in association with the index endometrial cancer cases were identified in subsets of patients. The disease associations included first and/or second degree relatives with breast, ovarian, endometrial and colorectal cancer. No relationships between the presence of the 870 G>A SNP and endometrial cancer in association with family clusterings of breast, ovarian or endometrial cancer were observed. There was, however, a significant association between the cyclin D1 870 G>A polymorphism and family history of colorectal cancer shown in table 3. Endometrial cancer patients with the variant (AA) genotype had a higher frequency of family members with colorectal cancer compared to those with the GG and GA genotypes (GG versus AA; OR 2.951, 95% CI (1.026–8.491), p = 0.045, and GG+GA versus AA; OR 2.265, 95% CI (1.048–4.894), p = 0.038).

In regards to the other environmental and reproductive risk factors examined in the case group, the genotype fre-

Table 1: Comparison of Environmental and Reproductive Risk Factors between Cases and Controls

Risk Factor	Group	Cases n (%)	Controls n (%)	OR	95% CI	P value
High Blood Pressure (yes/no)	yes	107 (56.0)	114 (39.2)	1.978	1.366 – 2.864	p < 0.001
	no	84 (44.0)	177 (60.8)			
Diabetes (yes/no)	yes	44 (23.0)	31 (10.7)	2.51	1.519 – 4.148	p < 0.001
	no	147 (77.0)	260 (89.3)			
Hormone Therapy (yes/no)	yes	47 (24.6)	40 (13.7)	2.048	1.282 – 3.273	p = 0.003
	no	144 (75.4)	251 (86.3)			
Alcohol consumption (ever/never)	ever	92 (48.2)	228 (78.4)	0.257	0.172 – 0.382	p < 0.001
	never	99 (51.8)	63 (21.6)			
BMI	<25 kg/m^2	34 (19.1)	72 (24.7)	0.718	0.454–1.136	p = 0.157
	>= 25 kg/m^2	144 (80.9)	219 (75.3)			
Smoking (ever/never)	ever	52 (27.2)	68 (23.4)	1.227	0.807 – 1.865	p = 0.338
	never	139 (72.8)	223 (76.6)			
Ovarian Cancer (yes/no)	yes	7 (3.7)	1 (0.3)	11.033	1.346 – 90.403	p = 0.025
	no	184 (96.3)	290 (99.7)			
Cervical Cancer (yes/no)	yes	8 (4.2)	2 (0.7)	6.317	1.327 – 30.077	p = 0.021
	no	183 (95.8)	289 (99.3)			
History of Ovarian or Cervical Cancer (yes/no)	yes	15 (7.9)	3 (1.0)	8.182	2.335 – 28.663	p = 0.001
	no	176 (92.1)	288 (99.0)			
Personal History of Any Cancer (yes/no)	yes	51 (26.7)	28 (9.6)	3.422	2.066 – 5.667	p < 0.001
	no	140 (73.3)	263 (90.4)			
History of Skin Cancer (yes/no)	yes	20 (10.5)	19 (6.5)	1.674	0.869 – 3.228	p = 0.124
	no	171 (89.5)	272 (93.5)			
History of Bowel Cancer (yes/no)	yes	10 (5.2)	8 (2.7)	1.954	0.757 – 5.045	p = 0.166
	no	181 (94.8)	283 (97.3)			

Note: BMI not known for 13 cases.

quencies of the cyclin D1 870 G>A polymorphism showed no further significant results.

Discussion

It is well known that endogenous and exogenous estrogen is implicated in endometrial cancer etiology since their products and metabolites have the ability to cause DNA damage. Given the importance of cell cycle control for the maintenance of genomic integrity, it is conceivable that polymorphisms in the genes that drive these processes alter the efficiency of DNA repair, leading to disease initiation. The current study took into account a series of environmental and reproductive risk factors for both the cases and controls and the results support previous epidemiological data for the listed risk factors indicating that there were no unusual environmental characteristics associated with the study population.

This study focused on the 870 G>A polymorphism in cyclin D1 and its association with endometrial cancer risk. The 870 G>A polymorphism was not significantly associated with endometrial cancer risk. However, a trend

Table 2: Association of Cyclin D1 870 G>A (rs603965) Polymorphism with Endometrial Cancer Risk

Genotype	Cases n (%)	Controls n (%)	χ^2	OR (95% CI) and p value	
GG	49 (25.7)	93 (32.1)	p = 0.161	1.00 (reference)	
				1.262 (0.771–2.066)$_{adj\#}$	p = 0.355
				1.256 (0.815–1.938)	p = 0.302
AA	48 (25.1)	55 (19.0)		1.692 (0.939–3.049)$_{adj\#}$	p = 0.080
				1.656 (0.989–2.784)	p = 0.057
GA+AA	142 (74.3)	197 (67.9)	p = 0.131†	1.383 (0.869–2.201)$_{adj\#}$	p = 0.172
				1.368 (0.910–2.057)	p = 0.132
GG+GA	143 (74.9)	235 (81.0)	p = 0.107‡	1.458 (0.889–2.393)$_{adj\#}$	p = 0.135
				1.434 (0.924–2.226)	p = 0.108

Note: Minor Allele Frequency (MAF) 0.317.
OR_{adj}: Adjusted for BMI, HBP, diabetes, HT, personal history of cancer, smoking and alcohol use.
† p value: Wild type genotype (GG) compared to combination of heterozygous and homozygous variant genotypes (GA+AA).
‡ p value: Homozygous variant genotype (AA) compared to combination of wild type and heterozygous genotypes (GG+GA).

Table 3: Association of Cyclin D1 870 G>A (rs603965) Polymorphism and Family History of Colorectal Cancer in Endometrial Cancer Cases

Genotype	FH of Colorectal Cancer in Endometrial Cancer Cases (n = 191)		χ^2	OR (95% CI) and p value	
	Yes n (%)	No n (%)			
GG	6 (3.1)	43 (22.5)	p = 0.084	1.00 (reference)	
GA	16 (8.4)	78 (40.8)		1.470 (0.536–4.034)	p = 0.454
AA	14 (7.3)	34 (17.8)		***2.951 (1.026–8.491)***	***p = 0.045***
GA+AA	30 (15.7)	112 (58.6)	p = 0.170#	1.920 (0.747–4.936)	p = 0.176
GG+GA	22 (11.5)	121 (63.4)	***p = 0.035***†	***2.265 (1.048–4.894)***	***p = 0.038***

Note: FH, family history.
p value: Wild type genotype (GG) compared to combination of heterozygous and homozygous variant genotypes (GA+AA).
† p value: Homozygous variant genotype (AA) compared to combination of wild type and heterozygous genotypes (GG+GA).

towards the AA genotype imparting an increased risk of endometrial cancer compared to the GG genotype was observed. These results support the study of Kang et al. (2005) [12] who found a significant association between the AA genotype and an increased risk in endometrial cancer development in Korean women (GG+GA versus AA, OR_{adj} 2.63 (1.04–6.66), p = 0.041) and suggests that this polymorphism is acting as a disease modifier. Even though we did not observe a statistically significant result, the underlying biological plausibility remains consistent between the two studies. The most likely explanation for the difference in the significance of the results between the current study and that of Kang et al. (2005) [12] is the influence of different environmental factors affecting disease risk. A number of studies have shown that overexpression of cyclin D1 is associated with endometrial cancer and that it is an early event in tumourigenesis [3,4,6-9]. One report showed that 50% (7/14) of endometrial carcinomas had cyclin D1 overexpression and that there was no immunopositive difference between these carcinomas and simple hyperplasia which is a precursor for endometrial cancer development [3].

From the study participants two main familial associations were observed, one with colorectal cancer (36 endometrial cancer patients (18.8%) with family history of colorectal cancer) and the other with breast cancer (44 endometrial cancer patients (23.0%) with family history of breast cancer). Interestingly, analysing family history of colorectal cancer in patients with endometrial cancer and the cyclin D1 870 G>A polymorphism showed that endometrial cancer patients with the AA genotype had a higher frequency of family members with colorectal cancer compared to endometrial cancer patients with the GG or GA genotype combinations.

The association between endometrial cancer and colorectal cancer is well recognised in Lynch Syndrome (or HNPCC); however, endometrial cancer associations with other malignancies are less well defined and there is little written in the literature about breast/endometrial cancer familial clustering. No association was observed between the cyclin D1 G870A SNP and endometrial/breast cancer families whereas an association was observed between this polymorphism and the increased likelihood of colorectal cancer in other family members.

There have been a number of studies that have focused on the cyclin D1 870 G>A polymorphism and colorectal cancer development however these reports are somewhat conflicting [16-18]. Some studies have reported an association between the cyclin D1 870 G>A polymorphism and the age of diagnosis of colorectal disease in HNPCC [19-21]. Endometrial cancer is the most common cancer in women with HNPCC and the second most common cancer overall in this syndrome [22], therefore the association of the cyclin D1 870 G>A polymorphism and a family history of colorectal cancer is intriguing and suggests that this polymorphism may be related to the increased risk of endometrial cancer in HNPCC.

This study has a number of strengths since it was a population-based case-control investigation that had a relatively large sample size to detect an association and detailed information of specific risk factors. However, these results must be confirmed in a larger population and more studies should be conducted on the association between the cyclin D1 870 G>A polymorphism and endometrial cancer risk. Additionally, the relationship between the cyclin D1 870 G>A polymorphism and HNPCC, specifically hMSH2 mutation carriers, should also be confirmed in further studies.

In conclusion, we have shown that the 870 G>A polymorphism in cyclin D1 may be associated with endometrial cancer risk and provided support for an association with colorectal cancer risk.

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Table III: Author Contribution Percentage and Description of Contribution to the Article Listed Above

Author	Contribution %	Description of Contribution to Article	
Katie A. Ashton	75%	Designed and executed the study. Provided significant insight into the interpretation of the data. Wrote the manuscript.	
Anthony Proietto	5%	Supplied samples and clinical information.	
Geoffrey Otton	5%	Supplied samples and clinical information.	
Ian Symonds	5%	Contributed to the design of the study.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected the manuscript.	

Chapter VII

Genetic variants in MUTYH are not associated with endometrial cancer risk

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Genetic variants in MUTYH are not associated with endometrial cancer risk

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Abstract

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is an autosomal dominant inherited predisposition to a number of epithelial cancers, most notably colorectal and endometrial cancer. Outside of the context of Lynch syndrome there is little evidence for an autosomal dominant or recessive condition that predisposes to endometrial cancer. Recently, genetic variants in MUTYH have been associated with a recessive form of colorectal cancer, known as MUTYH associated polyposis or MAP. MUTYH is involved in base excision repair of DNA lesions and as such a breakdown in the fidelity of this process would necessarily not be predicted to result in a specific disease. At present there is little information about the role of MUTYH in other types of cancer and only one report indicating a possible relationship with endometrial cancer.

Similar to a previous study, we investigated a series of endometrial cancer patients to determine if MUTYH variants were over-represented compared to a series of healthy control subjects and to assess whether or not endometrial cancer risk could be explained by an autosomal recessive model of inheritance.

Two MUTYH mutations, Y165C and G382D, and three common MUTYH polymorphisms, V22M, Q324H and S501F, were genotyped in 213 endometrial cancer patients and 226 controls from Australia using real time PCR. Differences in genotype frequencies were compared using Chi-squared analysis and by calculating odds ratios and 95% confidence intervals.

Three endometrial cancer patients were identified with heterozygous MUTYH mutations (two G382D and one Y165C). No bi-allelic mutation carriers were identified. Two of the three patients' clinical characteristics were similar to those commonly identified in HNPCC and lend support to the notion that MUTYH mutations increase the risk of developing HNPCC related diseases. There was no difference in the five genotype frequencies of the endometrial cancer patients compared to the controls. The results of our study suggest that MUTYH is unlikely to be involved in the genetic basis of endometrial cancer but a possible association of MUTYH variants with HNPCC related diseases cannot be excluded.

Background

MUTYH (MYH) is a DNA glycosylase which plays an essential role in the base excision repair (BER) pathway to prevent the accumulation of mutations that are a result of oxidative DNA damage [1]. In 2002, two autosomal recessive inherited mutations in MUTYH, Y165C and G382D, were associated with adenomatous polyposis and colorectal cancer [2], and several additional studies have confirmed that bi-allelic mutation carriers have an increased risk of developing colorectal cancer [3-5]. These two mutations are reported to account for approximately 86% of all variations in the MUTYH gene that are identified in Caucasians [6].

Some studies have suggested that mono-allelic changes in MUTYH increase colorectal cancer risk however this remains to be confirmed [3,4]. Furthermore, association studies assessing mono-allelic changes in MUTYH in combination with DNA mismatch repair genes, have revealed a possible relationship, specifically between hMSH2 and hMSH6, and an increased risk of developing colorectal cancer although this remains to be definitively confirmed [7,8]. Mutations in MMR genes are associated with hereditary non-polyposis colorectal cancer (HNPCC) which is an autosomal dominant inherited predisposition to colorectal cancer, endometrial cancer and a number of other malignancies [9]. Results from previous studies point towards a role of MUTYH mutations in HNPCC and suggest that they may be involved in a larger spectrum of disease involving extra-colonic cancers [7,8].

The role of MUTYH mutations in extra-colonic cancers has previously been reviewed and there are indications that this gene is associated with a broader spectrum of disease [6]. The report by Barnetson et al. (2005) focused on determining whether or not variants in MUTYH were related to endometrial cancer risk [6]. They identified one patient that was a compound heterozygote for Y165C and G382D. This patient had a sebaceous carcinoma which is a feature of Muir-Torre syndrome and is associated with MMR gene mutations. Five patients heterozygous for either Y165C or G382D were also identified. These MUTYH heterozygous mutation carriers did not harbour other pathogenic mutations, only a number of intronic variants. Since their conclusion that bi-allelic changes may increase susceptibility to endometrial cancer is based on one patient, these results need to be confirmed in a larger number of endometrial cancer cases.

In addition to the common MUTYH mutations, Y165C and G382D, three common polymorphisms in the Caucasian population have been identified: V22M, Q324H and S501F [2]. These polymorphisms have been suggested as being associated with an increased risk of developing colorectal cancer, however, it remains to be determined if these changes are tissue specific with respect to disease risk. Notwithstanding, these five MUTYH variants represent a significant proportion of the genetic variation present in MUTYH and warrant further investigation.

To confirm if the two common MUTYH mutations, Y165C and G382D and three common polymorphisms, Q324H, V22M and S501F are associated with endometrial cancer, 191 endometrial cancer patients were genotyped for these 5 variants.

Materials and methods

Study population

This study initially consisted of 213 consecutively recruited women with histologically confirmed endometrial cancer who presented for treatment at the Hunter Centre for Gynaecological Cancer, John Hunter Hospital, Newcastle, New South Wales, Australia between the years 1992 and 2005. Women that had additionally been diagnosed with breast cancer were excluded from this study.

The final analysis included 191 endometrial cancer patients. Data on reproductive and environmental risk factors including ethnicity, body mass index (BMI), diabetes, high blood pressure (HBP), age of diagnosis of endometrial cancer, age of menarche, age of menopause, other personal cancer history, family cancer history (Family history of cancer was defined as cancer in the index patient plus one or more 1st or 2nd degree relatives diagnosed with cancer), parity, breastfeeding, oral contraceptive use, chemotherapy, radiotherapy, hormone replacement therapy (HRT), smoking and alcohol use was collected using self reported questionnaires. Information regarding recurrence, stage, grade and histology of endometrial cancer was collected from the medical records. Two healthy anonymous control populations were used in this study from the Hunter Area of Newcastle. DNA samples were collected between the years of 1993 and 1997. For the Q324H, V22M and S501F polymorphisms, 226 patients were genotyped, and for the Y165C and G382D variants, 120 patients were genotyped and had a mean age of 51 years.

All participants provided informed written consent prior to participation in this study. Ethics approval was obtained from the Human Research Ethics Committee, University of Newcastle and the Hunter Area Research Ethics Committee, Hunter New England Health Service, Newcastle, New South Wales, Australia.

DNA isolation

Genomic DNA was extracted from 10 ml EDTA blood as previously described [10].

Molecular analysis

Genotyping of the five MUTYH polymorphisms Y165C, G382D, S501F, Q324H and V22M were performed on an ABI PRISM® 7500 Real-Time PCR System (PE Applied Biosystems, Foster City, CA), using primers and probes from Assay-by-Demand (Q324H and V22M) (assay ID: Q324H – C___27504565_10 and V22M – C___25955644_10) and Assay-by-Design (Y165C, G382D and S501F) (Applied Biosystems). The primers and probes for Y165C, G382D and S501F are listed in table 1. All assays were performed under universal conditions previously described [11]. Briefly, the assay functioned under universal conditions with each reaction containing: 50 ng DNA, 0.125 μl 40× Assay Mix and 2.5 μl TaqMan® Universal PCR master mix made up to 5 μl with sterile water. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 92°C for 15 sec and 60°C for 1 min. Post PCR, the plate was scanned to allow discrimination between the different genotypes. The genotyping results were confirmed by a second laboratory research assistant and 5% of the samples were re-genotyped with 100% concordance. Any sample where a genotype could not be accurately assessed was re-genotyped. If it failed a second time, it was discarded from the analysis. The overall call rates were in the range from 96.0–100%.

Statistical analysis

To determine differences in genotype frequencies between the cases and controls, chi-squared (χ^2) statistics and odds ratios and 95% CIs were calculated. T-tests were used to determine differences in the age of diagnosis of endometrial cancer by genotype. The significance levels of all tests were set at $p < 0.05$ and were two-sided. All statistical analysis was performed with Intercooled STATA 8.2 (Stata Corp., College Station, TX, USA), SPSS Version 15 (SPSS Inc. Chicago, IL, USA).

Results

The genotype frequencies were compared between the cases and controls for the two MUTYH mutations and the three MUTYH polymorphisms however no significant differences were observed (see table 2). We included endometrial cancer cases that were likely to be a result of tamoxifen treatment as they had previously been diagnosed with breast cancer. These patients did not alter the genotype frequency results for the three polymorphisms nor did these patients have a mutation in Y165C or G382D.

For the pathogenic MUTYH mutations, Y165C and G382D, there were only 3 patients identified with heterozygous changes, 2 for G382D and 1 for Y165C. No bi-allelic changes were identified. Additionally, these women did not harbour any of the three common polymorphisms, V22M, Q324H or S501F. The characteristics of the MUTYH Y165C and G382D heterozygous mutation carriers are in table 3. Two of these patients had family histories of cancer that are possibly associated with a diagnosis of Lynch Syndrome as they both had first degree relatives with colorectal cancer. The other patient did not have any family history of disease.

T-tests were used to evaluate the influence of the five MUTYH polymorphisms on the age of diagnosis of endometrial cancer. No significant differences were observed (data not shown).

Discussion

Currently, patients with multiple colorectal adenomas, no APC gene mutation or HNPCC related colorectal cancer and no MMR gene mutation are recommended to undergo testing for germline mutations in MUTYH. It is not known whether mutations or polymorphisms in MUTYH are specific for colorectal cancer or if they encompass a larger spectrum of disease, especially that which is over-represented in Lynch Syndrome. Since the MMR genes, hMSH2 and hMSH6 are associated with HNPCC and evidence suggests that they directly interact with MUTYH, it is possible that MUTYH mutations are related to HNPCC extra-colonic cancers, specifically endometrial cancer.

This study identified three endometrial cancer patients with heterozygous Y165C or G382D changes in MUTYH. No bi-allelic mutation carriers were identified in 191 endometrial cancer patients, however the monoallelic

Table 1: Real-Time PCR Assay-by-Design Primers and Probes for MUTYH Y165C, G382D and S501F

SNP	Forward	Reverse	Wild Type Probe	Mutant Probe
MUTYH S501 (C>T)	CAGCCTTCCAAAAGGTTCCCA	GCTGTGTGCATCAGTGGAGAT	VIC-CACGGA<u>G</u>AGGACAC	FAM-CACGGA<u>A</u>AGGACAC
MUTYH Y165C (A>G)	CCACAGGAAGGTGAATCAACTCT	CCTTACCTTCCGAGCTCCCT	VIC-CTGGGCT<u>A</u>CTATTCT	FAM-GGGCT<u>G</u>CTATTCT
MUTYH G382D (G>A)	GACCCCTGCCTGGCT	GACGGGAACTCCCACAGT	VIC-CCTCTCAG<u>G</u>TCTGCTG	FAM-CCTCTCAG<u>A</u>TCTGCTG

Note: The mutation/polymorphism is underlined in the probe sequences.
* The MUTYH S501F polymorphism is designed on the reverse strand.

Table 2: The five MUTYH variants and their association with endometrial cancer risk

Polymorphisms	Genotype	Cases n (%)	Controls n (%)	X^2	OR (95% CI) and p value
MUTYH Y165C (A>G)	AA	190 (99.5)	120 (100)		1.00 (reference)
	AG	1 90.5)	0 (0.0)	P = 0.43	0.53 (0.02–13.1) p = 0.43
	GG	0 (0.0)	9 (0.0)		
MUTYH G382D (G>A)	GG	189 (99.0)	118 (98.3)		1.00 (reference)
	GA	2 (1.0)	2 (1.7)	p = 0.6	41.6 (0.22–11.5) p = 0.63
	AA	0 (0.0)	0 (0.0)		
MUTYH Y22M (G>A)	GG	172 (90.1)	194 (85.8)		1.00 (reference)
	GA	17 (8.9)	31 (13.7)		1.62 (0.86–3.02) p = 0.17
	AA	2 (1.0)	1 (0.4)		0.44 (0.04–4.94) p = 0.92
MUTYH Q324H (G>C)	GG	109 (57.1)	129 (59.2)		1.00 (reference)
	GC	71 (37.2)	75 (34.4)	p = 0.83	0.89 (0.59–1.35) p = 0.66
	CC	11 (5.8)	14 (6.4)		1.08 (0.4&-2.47) p = 0.86
MUTYH S501F (C>T)	CC	187 (97.9)	210 (96.8)		1.00 (reference)
	CT	4 (2.1)	7 (3.2)	p = 0.48	1.56 (0.45–5.41) p = 0.69
	TT	0 (0.0)	0 (0.0)		

mutation carriers could possibly have other rare alterations that were not investigated. Our results are similar to a previous study which found one bi-allelic mutation carrier and five patients with heterozygous changes [6]. Two of the three patients with heterozygous changes had a family history of colorectal and endometrial cancer which could possibly reflect their relationship with HNPCC.

We analysed three additional common polymorphisms in MUTYH, Q324H, V22M and S501F, but did not find a second variant in the women with heterozygous Y165C or G382D mutations. It is highly unlikely that these women harbour another as yet unidentified polymorphism since the analysis of these five polymorphisms accounts for a large majority of genetic variation in MUTYH in Caucasians. Additionally, we compared the genotype frequencies for all five MUTYH variants but did not find a significant difference between the endometrial cancer group and the controls which suggests that these variants do not appear to increase the risk of developing endometrial cancer, although a larger population is required to confirm this statement.

Table 3: Characteristics of MUTYH Y165C and G382D heterozygous mutation carriers

Characteristics	Y165C – patient 1	G382D – patient 2	G382D – patient 3
Year of Birth	1933	1935	1951
BMI (kg/m^2)	>30	25–30	25–30
Age of Diagnosis of Endometrial cancer (years)	71	70	52
Age of Menarche	15	16	15
Age of Menopause	55	45	52
No. of Children	0	3	2
Oral Contraceptive	Never Use	Never	Never
Other Diseases	High Blood Pressure Ovarian Cancer Colorectal Cancer	High Blood Pressure Diabetes	Diabetes
Family History of Sister Cancer	Colorectal cancer Mother & Maternal Aunt Breast Cancer	Father Colorectal Brother Leukaemia	None
Stage of Cancer	Unknown	1B	Mixed Mullerian Malignant Tumour
Grade of Cancer	1	1	3
Histology	Adenocarcinoma	Adenocarcinoma	Mixed Mullerian Malignant Tumour
Recurrence	None	None	None
Smoker	Never	Current	Never
Alcohol	Non-Drinker	Non-Drinker	Non-Drinker

Our results suggest that the MUTYH mutations, Y165C and G382D, only account, if at all, for a minority of endometrial cancer cases. We can not rule out the possibility that these mutations may act as modifiers of disease penetrance since they both have been predicted to interact functionally with hMSH2 and hMSH6. Furthermore, it is not clear whether there are tissue specific differences in disease expression that may be related to environmental influences that are specific for each anatomical site. Recently, a study of over 600 breast cancer cases also revealed similar results to our own in that no bi-allelic mutations were identified and there was no change in the risk of disease in mono-allelic carriers. Together, the combined results suggest that MUTYH is not associated with an increased risk of breast cancer [12] or endometrial cancer.

In conclusion, our results in combination with Barnetson et al. (2005) [6] reveal that variation in MUTYH is very limited in endometrial cancer and does not appear to alter the susceptibility to sporadic endometrial cancer however MUTYH variants possibly have some role in HNPCC related disease.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KAA carried out the molecular genetic studies and wrote the first drafts of the manuscript. AP, GO and IS contributed to the collection and clinical histories of the patients. RJS conceived the study and participated in its design and coordination. All authors have read and approved the final version of the manuscript.

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Chapter VIII

General Discussion

Endometrial cancer is the fourth most common cancer in Australian women after lung, colorectal and breast cancer (Bray *et al.* 2005; AIHW 2007). Endogenous and exogenous risk factors associated with endometrial cancer are related to excessive or unopposed estrogen exposure (Akhmedkhanov *et al.* 2001). Epidemiological studies have highlighted that disease risk is not only dependent on environmental factors but is also influenced by genetic variation influencing these factors (Mahoney 2007; Meyer *et al.* 2008). Little is known about genetic predispositions to endometrial cancer apart from its association with the cancer family syndrome, hereditary non-polyposis colorectal cancer (HNPCC) (Lynch *et al.* 1993). It is, therefore, important to further our knowledge about the relationship between genetic variants and endometrial cancer risk. Understanding the variation in the genetic pathways that alter the likelihood of developing endometrial cancer is important for the identification of women at high risk, the improvement of treatment and patient outcomes, and elucidating the molecular mechanisms that underlie this disease.

In the human genome, there is only a small amount of variation in the genome between individuals. Genetic differences that alter the functionality of key proteins either by altering gene expression or by change of function are the source of variability that is likely to affect our susceptibility to disease (Brookes 1999; Wright 2005). Studying polymorphisms in polygenic diseases, such as endometrial cancer, has recently become a major focus in order to identify pathways involved cancer initiation and progression (Meyer *et al.* 2008). Phenotypic presentation may represent several different molecular mechanisms that result in a single poorly differentiable phenotype.

For the study presented herein, the candidate gene approach was used to identify genetic risk factors associated with endometrial cancer. Variations in the pathways involving hormone biosynthesis, hormone receptors and estrogen metabolism were studied to determine their association with disease risk. In addition, polymorphisms in the DNA repair and cell cycle control pathways, involved in the recognition and repair of estrogen-induced DNA lesions, were examined to establish their association with endometrial cancer risk.

This thesis focused on two main aspects of endometrial carcinogenesis:

i. HNPCC related endometrial cancer and genetic variation in estrogen metabolism.

ii. Polymorphisms in hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair, cell cycle control and risk of endometrial cancer.

8.1 HNPCC Related Endometrial Cancer and Genetic Variation in Estrogen Metabolism

The first study of this thesis, described in chapter 2, examined an Australian and Polish HNPCC cohort to determine if the COMT V158M polymorphism was responsible for altering disease expression. The results of this study revealed a significant association of the COMT V158M polymorphism and risk of endometrial/ovarian cancer in MMR mutation negative women. COMT is a phase II estrogen metabolising enzyme that is important for the inactivation of catechol estrogens that are formed during phase I estrogen metabolism (Dawling *et al.* 2001). The COMT V158M polymorphism decreases the activity of the enzyme in the heterozygous and homozygous variant states, and consequently, COMT does not efficiently inactivate catechol estrogens that have the ability to cause DNA damaging events (Zhu 2002). Endometrial and ovarian cancer are part of the HNPCC disease spectrum (Schmeler *et al.* 2008) and are influenced by excessive or unopposed estrogen exposure, therefore, the COMT V158M polymorphism may, by virtue of altering the kinetics of estrogen metabolism, account for a proportion of women with HNPCC associated endometrial/ovarian cancer.

The women with endometrial/ovarian cancer had a higher frequency of the heterozygous genotype and a lower frequency of the homozygous mutant genotype in comparison to women without endometrial/ovarian cancer. A high frequency of the heterozygous genotype is indicative of heterosis, where there is an apparent greater effect of the heterozygous state (Comings *et al.* 2000). Heterosis can occur at polymorphic loci when the relative fitness of the heterozygotes is either greater or less than that of the homozygotes, known as positive or negative heterosis, respectively (Comings *et al.* 2000). The results show negative heterosis; however, the exact mechanisms underlying this phenomenon are not clear. One explanation suggests that optimal activity may be occurring at the homozygous ends rather than the expected

high, medium and low gene expression associated with the three genotypes, wild-type, heterozygous and homozygous variant (Comings *et al.* 2000). In addition, there may be other unknown factors causing stratification of the results, favouring the heterozygous genotype (Comings *et al.* 2000). Clearly, this association must be investigated further.

There are no previous studies examining variation in estrogen metabolism and risk of endometrial/ovarian cancer in HNPCC women. This result is novel, indicating that the COMT V158M polymorphism may alter the risk of developing endometrial/ovarian cancer in MMR mutation negative HNPCC women. HNPCC is a specific clinico-pathological entity, therefore, closer examination of these findings were required to determine whether polymorphisms in estrogen related pathways; hormone biosynthesis, hormone receptors and estrogen metabolism, were related to endometrial cancer development, outside of the context of an inherited predisposition to disease. Notwithstanding, estrogen and its metabolites have the ability to contribute to estrogen-induced cancer through the formation of DNA adducts and reactive oxygen species (ROS), therefore variation in the genes controlling DNA damage were also examined.

8.2 Polymorphisms in Hormone Biosynthesis and Hormone Receptors Genes and Risk of Endometrial Cancer

Hormone biosynthesis involves the production of estrogen, progesterone and testosterone, catalysed by a network of enzymes. By binding to their respective receptors, steroid hormones induce specific physiological responses. Perturbations in the genes involved in the production of hormones; CYP11A1, CYP17A1, CYP19A1, and HSD17β1, or the hormone receptors; estrogen receptor alpha (ESR1), estrogen receptor beta (ESR2), progesterone receptor (PGR), amplified in breast cancer I (AIB1) and the androgen receptor (AR), were assessed to determine their association with endometrial cancer risk.

In chapter 3, polymorphisms in the four hormone biosynthesis genes, CYP11A1, CYP17A1, CYP19A1 and HSD17β1 were not associated with an altered risk of developing endometrial cancer, suggesting that the production of hormones in women with endometrial cancer, are not compromised by polymorphisms in this pathway. Our results are in concordance with the majority of studies that have examined variations in these genes and the risk of endometrial cancer (Haiman *et al.* 2001; McKean-Cowdin *et al.* 2001; Berstein *et al.* 2004; Setiawan *et al.* 2004; Paynter *et al.* 2005; Aban *et al.* 2006; Szyllo *et al.* 2006; Gaudet *et al.* 2008; Hirata *et al.* 2008). Taken together, the results reveal that the processes involving the production of

hormones are not implicated in endometrial cancer risk; rather the effect of the hormones and their metabolism may be involved in disease initiation and development. This point is highlighted in chapters 3 and 4. The results revealed that polymorphisms in the estrogen receptors (ESR1 and ESR2) and the androgen receptor (AR) were significantly associated with endometrial cancer risk.

8.2.1 Estrogen receptors (ESR1 and ESR2)

Four polymorphisms in estrogen receptor alpha (ESR1); rs2234693, rs9340799, TA(n) and GT(n), and two polymorphisms in estrogen receptor beta (ESR2); rs1255998 and rs944050, were associated with an increased risk of developing endometrial cancer. Although the functional significance of these polymorphisms is not yet clear, a number of investigators that have studied these variants and the risk of developing other types of cancers, have suggested that these polymorphisms may affect the kinetics of estrogen binding to these receptors thereby permitting increased levels of circulating estrogen (del Senno *et al.* 1992; Yaich *et al.* 1992; Roodi *et al.* 1995; Sand *et al.* 2002; Iwamoto *et al.* 2003; Maguire *et al.* 2005; Beleza-Meireles *et al.* 2006; Sowers *et al.* 2006). Excessive estrogen has been shown to be detrimental to the cells of the endometrium as it has the ability to cause the formation of DNA lesions, leading to the initiation of endometrial cancer (Henderson *et al.* 2000; Liehr 2000).

8.2.2 Androgen Receptor (AR)

A highly polymorphic CAG trinucleotide repeat, located in the promoter region of the androgen receptor was associated with endometrial cancer risk. The androgen receptor is a transcription factor that regulates the transactivation of a number of hormone responsive genes (Tilley *et al.* 1989). Previous studies have demonstrated that shorter CAG repeats of the AR are related to higher transcriptional activity (Chamberlain *et al.* 1994; Choong *et al.* 1996). The results presented in this thesis indicated that a higher number of CAG repeats were related to a decreased risk of developing endometrial cancer. A higher number of CAG repeats, decreases the transcription of hormone responsive genes, thereby preventing excessive production of estrogen (Chamberlain *et al.* 1994; Choong *et al.* 1996), and appears to be protective for the development of endometrial cancer as there is a decrease in hormone production. This result is in concordance with another study (McGrath *et al.* 2006) and confirmed the association that a higher number of CAG repeat lengths in the AR are associated with decreased endometrial cancer risk.

These findings further demonstrate how estrogens and androgens can have profound effects on the growth and differentiation of the endometrium by virtue of a genetic predisposition. These findings have broad implications for the identification and implementation of prevention strategies for women at high risk of developing endometrial cancer.

8.3 Polymorphisms in Estrogen Metabolism Genes and Risk of Endometrial Cancer

Estrogens elicit a wide range of biological effects by binding to the estrogen receptor to induce the expression of genes involved in many important functions (Deroo *et al.* 2006). Estrogens are eventually detoxified and eliminated by a complex network of metabolising enzymes (Zhu *et al.* 1998).

Chapter 3 examined 13 polymorphisms in 9 genes involved in phase I and II of estrogen metabolism and the risk of endometrial cancer. Analysis of the polymorphisms separately revealed that the variant genotypes of both the GSTM1 deletion and CYP1B1 rs1800440 were associated with a decreased risk of developing endometrial cancer. Haplotype analysis also showed that specific combinations of variants in CYP1A1, CYP1B1 and the GSTs also conferred a decrease in endometrial cancer risk. Phase I estrogen metabolism involves the 2- or 4- hydroxylation of estrogens whereas phase II inactivates and eliminates catechol estrogens formed during phase I. The results showed that polymorphisms in genes involved in both phases of estrogen metabolism are implicated in endometrial cancer risk. In addition to estrogen-induced cancer, the dysfunction of the pathways involved in the metabolism of estrogen may offer a complementary genotoxic pathway mediated by the production of reactive catechol estrogens that can form lesions in the DNA and have the ability to generate reactive oxygen species (ROS) during redox cycling. Functional studies reported for the CYP1A1, CYP1B1, GSTM1 and GSTP1 polymorphisms suggest that they increase the formation of catechol estrogens or do not successfully detoxify these metabolites. Therefore, polymorphisms in these genes are expected to be associated with an increase in endometrial cancer risk rather than a decrease, as observed in this study.

The results for the CYP1B1, GSTM1 and GSTP1 polymorphisms may reveal an alternate mechanism of carcinogenesis. These enzymes are involved in the detoxification of xenobiotics and it cannot be ruled out that loss of enzymatic function of these genes is potentially a protective combination. Specifically for CYP1A1, the minor

allele frequency of the rs1048943 polymorphism in our case and control populations was quite low and the significant observation is possibly a type I statistical error. Other studies examining these variants and endometrial cancer risk have yielded inconsistent findings (Esteller *et al.* 1997; Sugawara *et al.* 2003; Doherty *et al.* 2005; Seremak-Mrozikiewicz *et al.* 2005; Esinler *et al.* 2006; Mikhailova *et al.* 2006; McGrath *et al.* 2007); therefore the results must be confirmed in a larger independent data set, to provide adequate power for the detection of true associations. Clearly, further investigation of all polymorphic variation in these genes and analysis of gene-gene interactions is required as the association studies involving these genes and endometrial cancer risk are still inconclusive.

The work comprised in this section of the thesis, showed that variation in genes involved in both phases of estrogen metabolism appears to alter the risk of developing endometrial cancer. In addition to the estrogen and androgen receptor findings described earlier, perturbations in phase I and II estrogen metabolism may aid in the identification of women at high risk of developing endometrial cancer.

8.4 Polymorphisms in DNA Repair and Cell Cycle Control Genes and Risk of Endometrial Cancer

Estrogen induces the proliferation of cells within the endometrium; however excessive exposure to estrogen increases individual susceptibility to genetic errors that may occur during DNA replication. As a consequence, the cells are given the opportunity to accumulate random genetic errors (Enomoto *et al.* 1991; Lord *et al.* 2002). To maintain genomic integrity, many processes involved in cell cycle control and DNA repair are essential for the recognition, initiation and repair or elimination of damage that occurs to the cells of the endometrium to maintain the fidelity of the genetic code.

There are a large number of genes involved in these processes; however, the focus of this study was to examine polymorphisms in TP53 and MDM2 (chapter 5), Cyclin D1 (chapter 6) and MUTYH (chapter 7). Investigating polymorphisms in these genes and the risk of endometrial cancer failed to reveal any significant results, suggesting that variations in these specific DNA repair processes do not alter the risk of developing endometrial cancer.

The cyclin D1 870 G>A polymorphism showed a trend for an increase in endometrial cancer risk, similar to another study (Kang *et al.* 2005), but the results

were not significant, possibly due to the adjustment of endometrial cancer risk factors and the different ethnic population examined. Cyclin D1 is integral for the G1 to S phase of the cell cycle as it regulates cellular proliferation (Sherr 1996) however dysfunction of cyclin D1 due to the 870 G>A polymorphism, leads to uncontrollable cellular growth (Betticher *et al.* 1995; Hosokawa *et al.* 1998) and potentially the initiation of endometrial cancer. Further studies are required on a larger, independent population to assess whether variation in DNA repair and cell cycle control is associated with endometrial cancer. It cannot be excluded that polymorphisms in other cell cycle control and DNA repair genes may be involved in the risk of endometrial cancer.

Interestingly, stratification of the cases revealed that the combination of the TP53 and MDM2 polymorphisms were associated with a high grade of endometrial cancer. Polymorphisms in these genes cause TP53 inactivation and MDM2 over-expression, leading to the accumulation of genetic errors (Wu *et al.* 2002; Bond *et al.* 2004) and consequently reducing the ability of damaged cells to apoptose. The results of this study are in concordance with a previous study (Saffari *et al.* 2005) and add further weight to the hypothesis that reduced apoptotic ability is associated with higher grades of endometrial cancer, and as a consequence, lower survival rates. This result has the potential to have significant therapeutic implications for women at risk of developing endometrial cancer. Women with reduced apoptotic ability, due to combinations of polymorphisms in TP53 and MDM2, may be given the option of more aggressive or alternative treatments, as they have a significantly increased risk of presenting with higher grades of endometrial carcinomas, that are associated with lower survival rates.

8.5 Overall Conclusions

The study of genetic polymorphisms may help explain genetic differences in individual susceptibility to cancer and are markers of risk that aid in the development of effective and personalised strategies to prevent disease development. Most cancers are multifactorial, and result from the combination of environmental effects and DNA sequence variation. It is becoming increasingly evident that cancer susceptibility results from the additive effect of a number of genetic variants that individually contribute a modest change in disease risk (Meyer *et al.* 2008). Identification of these alterations may lead to the development of tests, aiding in the implementation of preventative strategies. This point can be highlighted by examining the significant reduction of

endometrial cancer in HNPCC women harbouring MMR gene mutations that undergo prophylactic hysterectomy (Lynch *et al.* 2007).

This study revealed the following associations:

i. The COMT V158M polymorphism may account for some of the women with HNPCC related endometrial/ovarian cancer that do not harbour MMR gene mutations.

ii. Polymorphisms in the estrogen and androgen receptors are associated with an increase and decrease in endometrial cancer risk, respectively.

iii. Polymorphisms in CYP1A1, CYP1B1, GSTM1 and GSTP1 are associated with a decrease in endometrial cancer risk.

iv. The cyclin D1 870 G>A polymorphism is possibly associated with an increased risk of developing endometrial cancer.

v. Combinations of polymorphisms in TP53 and MDM2 are associated with higher grades of endometrial cancer.

8.6 Recommendations

It has been well established that novel methods are required to risk-stratify women with endometrial cancer (Meyer *et al.* 2008). To date, there have been a small number of studies that have examined genetic variation in some genes involved in the pathways described in this thesis. This study is unique as it is the first to examine the major genes involved in hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair and cell cycle control and the most common genetic polymorphisms in these genes.

The candidate gene approach is an efficient and cost-effective method for the identification of genetic variants associated with disease based on prior knowledge of the biological mechanistic pathways. These types of studies are in a preliminary phase of being applied into larger and more diverse populations and point towards the notion that full candidate pathways must be analysed. As technology improves, the next step in this field of determining genetic variation associated with endometrial cancer risk is

performing whole genome genotyping. Full candidate pathways and whole genome genotyping require large sample numbers for validation of findings and these studies must be performed in multi-centre groups to provide adequate power to detect true associations.

This point is highlighted in recent studies involving prostate cancer. By performing whole genome genotyping, multiple genetic variants have been linked to three regions in 8q24 and alterations in prostate cancer risk (Amundadottir *et al.* 2006). One recent study analysed twelve polymorphisms within this defined region (Sun *et al.* 2008) and showed that specific regions harbouring different combinations of polymorphisms were associated with increased prostate cancer risk. Interestingly, one region in combination with a family history of prostate cancer provided strong evidence for an even greater increased risk of developing prostate cancer. Therefore, whole genome genotyping may identify a polymorphic region associated with endometrial cancer risk as seen in these recent studies of prostate cancer.

8.7 Summary

In conclusion, as illustrated in this thesis, the elucidation of the association between endometrial cancer and variation in genes involved in hormone biosynthesis, hormone receptors, estrogen metabolism, DNA repair and cell cycle control has contributed to the improved understanding of inter-individual differences relating to endometrial cancer risk.

Understanding the variation in the genes involved in these pathways and their relationship with endometrial cancer risk is crucial to reveal the mechanisms that contribute to the overall understanding of how endometrial cancer develops and thus identifies the specific alterations that are responsible for endometrial cancer initiation and progression. Finally, an increased understanding of genetic variation associated with cancer risk consistently improves not only our ability to understand complex diseases but also enhances our capacity to treat people with cancer at earlier stages to aid in better patient outcomes.

The End

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