



UNIVERSITY OF
LIVERPOOL

Endometrial cancer: involvement of androgens and metastasis inducing proteins

**Thesis submitted in accordance with the requirements
of the University of Liverpool for the degree of
Doctor in Philosophy**

By

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July 2016

Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree



Areege M Kamal

«قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا»

سوره الاسراء ايه (85)

“Say: the spirit is of the command of my Lord; and in no way have you been brought of knowledge except a little.”

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Acknowledgments

Thanks to God the most graceful most merciful who has blessed me and taught me things I did not know. I should acknowledge his mighty bless for placing me in the Women's department with this wonderful team who made the PhD a positively unforgettable experience.

First of all, I would like to express my deepest thanks and gratitude to my supervisor, Dharani, for her continuous support, help and priceless advice. Thanks for being there in the most stretching time for discussion, great suggestion and guidance. Thanks for all the groundwork of ethics writing, patient consenting and sample collection. Indeed, I am fortunate to work with such a considerate and encouraging person. I would also like to acknowledge Dr Trish Murray and Dr John Green my secondary supervisors for their support and guidance.

Great thanks to Jo, Anthony, Lisa, Jane, Sarah and Helen for all the advice and technical support they have given me throughout these four years. I am also thankful to Dr. Dong Barraclough in the UCD at the Royal Liverpool Hospital for training me on qPCR and Dr. Angela Midgley in the Department of Children Health at Alderhay for helping me with flow cytometry. Thanks to Dr. Yasmeen Mir and Hawida Shawki in the Department of Pathology at the Royal Liverpool Hospital for providing access to pathology blocks and for finding time in their extra-busy schedule to provide pathological advice and training. Many thanks to Dr. Decruze for providing the updated outcome data of endometrial cancer patients and to Mr Lane for his statistical advice.

I am particularly grateful to Dr Judith Bulmer (Newcastle), Dr Pierre Martin-Hirsch and Helen Stringfellow (Lancaster), Prof Philip Rudland and Dr Roger Barraclough for their valuable pathological, clinical and scientific discussion and sharing resources.

Many thanks to the PhD gang, Acher, Aby, Seham, Asma, Aysha, Bee and Rafah for making the office such a pleasant place to work. Thanks to MPhil and MRes students; Eve, Veda, Rya and Lucy who had helped in IHC staining as part of their own projects. Special thanks to Nic for stepping in to proofread this thesis and Meera for updating the outcome data at the very last minute.

I would like to acknowledge the Higher Committee for Education Development in Iraq for selecting me for this scholarship and for providing the financial support and continuous follow up.

I am indebted to my parents and parents in law for their endless love, support and encouragement were a great help during these years. There are no words to thank Zaid for his ceaseless patience and support. You have always been a source of hope at the moments of despair. Thanks to my sweeties Hasan and Ali for their patience throughout the four years. You have been really wonderful boys.

List of published papers

Papers published in peer-reviewed journals

1. Hapangama, D.K., **Kamal, A.M.**, Bulmer, J.N., 2015. Estrogen receptor β : the guardian of the endometrium. *Hum. Reprod. Update* 21, 174–193.
2. **Kamal, A.M.**, Tempest, N., Parkes, C., Alnafakh, R., Makrydima, S., Adishesh, M., Hapangama, D., 2016. Hormones and endometrial carcinogenesis. *Horm. Mol. Biol. Clin. Investig.* 25, 129–48.
3. **Kamal, A.M.**, Bulmer, J.N., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Hapangama, D.K., 2016. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. *Br. J. Cancer* 114, 688–96.
4. **Kamal, A.M.**, Tempest, N., Macane, A., Adishesh, M., Bhullar, J., Makrydima, S., Hapangama, D., Hormones interaction in endometrial cancer, in endometrial cancer mangement handbook by Springer. (submitted)
5. **Kamal, A.M.**, Tempest, N., Makrydima, S., Adishesh, M., Bhullar, J., Macane, A., Hapangama, D., Oestrogen receptors in the endometrium and endometrial cancer, in: *Advances in Experimental Medicine and Biology*” (AEMB) series by Springer. (submitted)

Manuscripts under preparation

1. **Kamal, A.M.**, Parkes, C., Valentijn, A.J., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Barraclough, D., Rudland, P.S., Hapangama, D.K. High AGR2 is a feature of Low Grade endometrial cancer and is potentially regulated by Androgen.
2. **Kamal, A.M.**, Parkes, C., Valentijn, A.J., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Barraclough, D., Rudland, P.S., Hapangama, D.K. The spatial and temporal expression of metastasis associated protein S100A4 and endometrial carcinogenesis.
3. Hapangama, D.K., **Kamal, A.M.**, Parkes, C., Valentijn, A.J., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Barraclough, D., Rudland, P.S. The role S100P protein in human endometrial cancer.
4. Parkes, C., **Kamal, A.M.**, Valentijn, A.J., Barraclough, D., Moss, D., Hapangama, D.K. Methodological guide for assessing molecular and functional features of Cell Lines in the context of endometrial cancer research.
5. Draper, H., **Kamal, A.M.**, Valentijn, A.J., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Hapangama, D.K. Prognostic value of SOX9 in endometrial cancer. (Submitted to Scientific Report)

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1. **Kamal, A.M.**, Raju, R., Homer, L., Drury, J., Barraclough, D., Hapangama, D. Metastasis Inducing Proteins Expression in Endometrial Cancer. Manchester Life Sciences PhD Conference, Manchester, UK, May 10th, 2013. (Poster presentation)
2. **Kamal, A.M.**, Palial, K., Drury, J., Valentijn, A., Hapangama, D. Loss of endometrial SSEA-1 expression is associated with human endometrial cancers. The CRUK Annual Meeting, Liverpool, UK, May, 21st, 2013. (Poster presentation)
3. **Kamal, A.M.**, Homer, L., Raju, R., Drury, J., Barraclough, D., DeCruze, S.B., Murray, P., Green, J., Hapangama, D. Proteins implicated in metastasis are upregulated in human endometrial cancer: potential prognostic indicators and therapeutic targets. ESGO 18, Liverpool, October, 13th, 2013. (Poster presentation)
4. **Kamal, A.M.**, Homer, L., Raju, R., Drury, J., Barraclough, D., DeCruze, S.B., Murray, P., Green, J., Hapangama, D. The expression of metastasis inducing proteins in endometrial cancer. North of England Cell Biology Forum, Liverpool, UK. September, 11th, 2013. (Poster presentation)
5. **Kamal, A.M.**, Bunni E, Avula V, Drury J, Hapangama D. Androgen receptors in normal and neoplastic endometrium: a detailed expression profile. Liverpool & Manchester Inter-University Postgraduate Symposium 2014 Physiology and Cell Biology, Liverpool, UK. April, 24th, 2014. (Poster presentation)
6. **Kamal, A.M.** Metastasis Inducing Proteins are Overexpressed in Human Endometrial cancer. North East Postgraduate Conference, Newcastle, UK. October, 31st, 2014. (Oral presentation)

7. **Kamal, A.M.** Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and are implicated in endometrial carcinogenesis. Liverpool Women's Great day, Liverpool, UK. December, 19th, 2014. (Oral presentation)
8. **Kamal, A.M.**, Bulmer, J.N., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Hapangama, D.K. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. British Gynaecological Cancer Society Annual Meeting, Newcastle, UK, July, 9th – 10th 2015. (Poster presentation)
9. **Kamal, A.M.**, Raju, R., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P., Hapangama, D.K. Upregulation of AGR2 is an early step in low grade endometrial carcinogenesis and progression. ESGO 19, Nice, France. October, 24th, 2015. (Poster presentation)
10. **Kamal, A.M.**, Parkes, C., Raju, R., DeCruze, S.B., Stringfellow, H.F., Martin-Hirsch, P. Hapangama, D.K. Epithelial S100A4 protein expression is a late event in endometrial carcinogenesis. ESGO 19, Nice, France. October, 24th, 2015. (Poster presentation)

List of abbreviations

A diol	Androstenediol
AGR2	Anterior gradient protein
Akt	Protein kinase B
APC	Adenomatous polyposis coli
AR	Androgen receptor
ASRs	Age-standardised incidence rates
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
CD14	Cluster of differentiation 14/ Monocyte differentiation antigen
CD45	Cluster of differentiation 45/ lymphocyte common antigen
CD68	Cluster of differentiation 68/ macrophage marker
cDNA	Complementary deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CNNB1	β catenin gene
COCP	Combined Oral Contraceptives
CS	Carcinosarcoma
CS-FBS	Charcoal stripped foetal bovine serum
CSS	Cancer specific survival
Ct	Cycle to Threshold
D/CDK4	Cyclin D/ Cyclin-dependent Kinase 4 complex
D/CDK6	Cyclin D/ Cyclin-dependent Kinase 6 complex
DAB	Diaminobenzidine
DES	Diethylstilbestrol
DFS	Disease free survival
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	5 alpha-dihydrotestosterone
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
E2	17 β -estradiol
EC	Endometrial cancer
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetra acetic acid
EH	Endometrial hyperplasia
EHA	Endometrial hyperplasia with cytological atypia
EHNA	Endometrial hyperplasia with no cytological atypia
EIC	Endometrial intraepithelial carcinoma
EMT	Epithelial mesenchymal transition
ER	Oestrogen receptor
ERE	Classical oestrogen response element
FACS	Fluorescence Activated Cell Sorting

FBS	Foetal bovine serum
FBXW7	F-Box and WD Repeat Domain Containing 7
FGF	Fibroblast growth factor
FIGO	International Federation of Gynaecology and Obstetrics
FSH	Follicular stimulating hormone
g	Relative Centrifugal Force
GC	Guanine and cytosine
GCP:	Good clinical practice
GnRH	gonadotrophic realising hormone
GOG	Gynaecologic Oncology Group
GPCR	G protein-coupled receptor
HGEC	High grade endometrial cancer
HSD17B7	17b-hydroxysteroid dehydrogenases
IGF-1	Insulin-Like Growth Factor-1
IGFR-1	Insulin-Like Growth Factor Receptor-1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
LGEC	Low grade endometrial cancer
LN	Lymph node
LNG-IUD	levonorgestrel intrauterine system
LREC	Local research ethics committee
LVSI	Lymphovascular space invasion
LWH	Liverpool Women's Hospital
MA	megestrol acetate
MMPs	Matrix Metalloproteinases
MPA	Medroxyprogesterone acetate
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
MUC1	Mucin1
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NBF	Neutral buffered formalin
OD	optical density
OS	Overall survival
P13KCA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PBS	Phosphate Buffered Saline
PCOS	Poly cystic ovarian syndrome
PD	Parkinson Disease
PI3K	phosphoinositide 3-kinase
POLE	Polymerase (DNA) Epsilon
preM	premenopausal
PM	postmenopausal
PP	Proliferative phase
PPP2R1A	Protein Phosphatase 2 Regulatory Subunit A, Alpha
PR	Progesterone receptor
PPIA	Peptidylprolyl Isomerase A

pRb	Phosphorylated retinoblastoma gene
PTEN	phosphatase and tensin homolog
RNA	Ribonucleic acid
RR	Relative risk
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
SD	Standard deviation
SEER	Epidemiology and End Results ()
SHBG	Sex hormone binding globulin
SP	Secretory phase
SSEA-1	Stage Specific Embryonic Antigen 1
TAE	Tris-Acetate EDTA
TBS	Tris-buffered saline
TGF-β	Transforming Growth Factor Beta
TP53	Tumour protein 53
VEGF	Vascular Endothelial Growth Factor
VEGFRs	Vascular Endothelial Growth Factor Receptors
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta
WHO	World Health Organisation

Abstract

Endometrial cancer: involvement of androgens and metastasis inducing proteins

Areege M. Kamal

The alarming escalation of endometrial cancer incidence with associated parallel rise in mortality has increased the urge to find reliable prognostic markers in order to improve risk stratification of patients and therefore promote more individualised treatment strategies. The principal goal of this thesis was to examine the prognostic role of steroid hormone receptors, particularly AR, and metastasis inducing proteins S100A4, S100P and AGR2 in endometrial cancer and to investigate the hormonal regulation of these proteins.

A comprehensive expression profile for each of these target proteins was described in a total of 161 well characterised human endometrial samples, using immunohistochemistry. The transcription of the target genes was further assessed in a subset of these samples using the RT-qPCR. Four established human endometrial cancer cell lines were then characterised for the expression of the steroid receptors and the metastasis inducing proteins, in order to select the most appropriate cell line for *in vitro* hormone modulation. Ishikawa cell line was subsequently utilised to study the effect of the potent natural androgen, 5 α -dihydrotestosterone (DHT), and oestradiol on the expression of the aforementioned target genes.

An immuno-scoring system was proposed and validated for more accurate quantification of steroid hormone receptor proteins in the endometrium. The expression of AR and ER α was significantly higher in the epithelium of postmenopausal endometrium compared with that of the premenopausal proliferative phase basalis layer. This expression pattern persisted in the hyperplastic epithelium, and also in low grade cancers. In contrast, the high grade cancers showed a significant loss of AR, PR and ER β compared with the low grade cancers, whilst maintaining weak to moderate ER α . Unlike PR, AR expression in metastatic lesions was significantly higher than that in primary tumours. AR expression correlated positively with favourable clinicopathological features and a lower proliferation index. Loss of AR with/without a simultaneous loss of PR was associated with significantly lower disease free, cancer specific and overall survivals. AR was an independent prognostic indicator for endometrial cancer progression.

S100A4, S100P and AGR2 proteins were upregulated in endometrial cancer. A substantial change in the expression profile of S100A4 was observed in endometrial cancer compared with the normal postmenopausal endometrium. The increased expression of S100A4 protein was represented by a significant

increase in the nuclear and cytoplasmic expression of the malignant epithelial cells with a concomitant high expression in the associated stroma. High immunoexpression of S100A4 was positively associated with deep myometrial invasion and shorter cancer specific and overall survival but was not identified as an independent prognostic indicator. The expression of S100P was generally low and limited to the cytoplasm of the normal postmenopausal glandular epithelium. Significant nuclear translocation was observed in endometrial cancer cells simultaneously with a reduction in cytoplasmic expression. The loss of cytoplasmic S100P (not nuclear) was associated with unfavourable prognostic indicators such as lymphovascular space invasion. Elevated AGR2 expression was observed in premalignant atypical hyperplasia and peaked in low grade cancers. There was a significant association between *high*AGR2 and positive ER α , PR, and AR in endometrial cancers. Secreted AGR2 was detected in the serum and uterine washes from endometrial cancer patients. AGR2 protein was immunoexpressed in 93/100 (93%) of the endometrial cancer samples and was associated with a longer overall survival of the patients.

In vitro studies on hormonal modulation showed that a supra-physiological dose of DHT was required to induce AR expression in Ishikawa cells. At such dose, DHT had a stimulatory effect on Ishikawa cell proliferation, an action exerted via ER. Whilst S100A4 and S100P mRNA level in Ishikawa cells did not show a significant change in response to oestradiol or DHT treatments, AGR2 mRNA level was downregulated after 24-hour treatment with DHT (but not oestradiol) which was, at least partially, via AR.

In conclusion, AR may be a clinically relevant prognostic indicator and a potential therapeutic target in endometrial cancer. Although S100A4 and AGR2 were not recognised as independent prognostic indicators, both have shown significant association with patient outcome. S100A4 protein can be potentially targeted by one of the newly developed S100A4 neutralising peptide to characterize its role in endometrial cancer invasion. An association between S100P and patient outcome was not identified; however, the change in the cellular localisation of S100P may have implications in endometrial carcinogenesis. DHT can modulate AR and AGR2 directly or via ER.

Chapter one

General Introduction

The endometrium is the inner lining of the uterus, its main function being to receive the conceptus and to maintain a successful pregnancy. Under the control of ovarian hormones, monthly cyclic morphological and functional changes occur in this dynamic tissue replacing the top layer with a fresh one in order to accommodate the upcoming fertilised ovum. This extraordinary regenerative function of the endometrium is preserved even after ovarian decline. Postmenopausal endometrium responds to exogenous steroid hormones and has the ability to regain proliferative function. A spectrum of hormone related proliferative conditions may occur in premenopausal endometrium yet endometrial cancer (EC) occurs predominantly after menopause. Molecular and hormonal aberrations play an important role in the neoplastic transformation of postmenopausal endometrium. When, why and how cancerous changes occur are the questions that scientists are trying to answer.

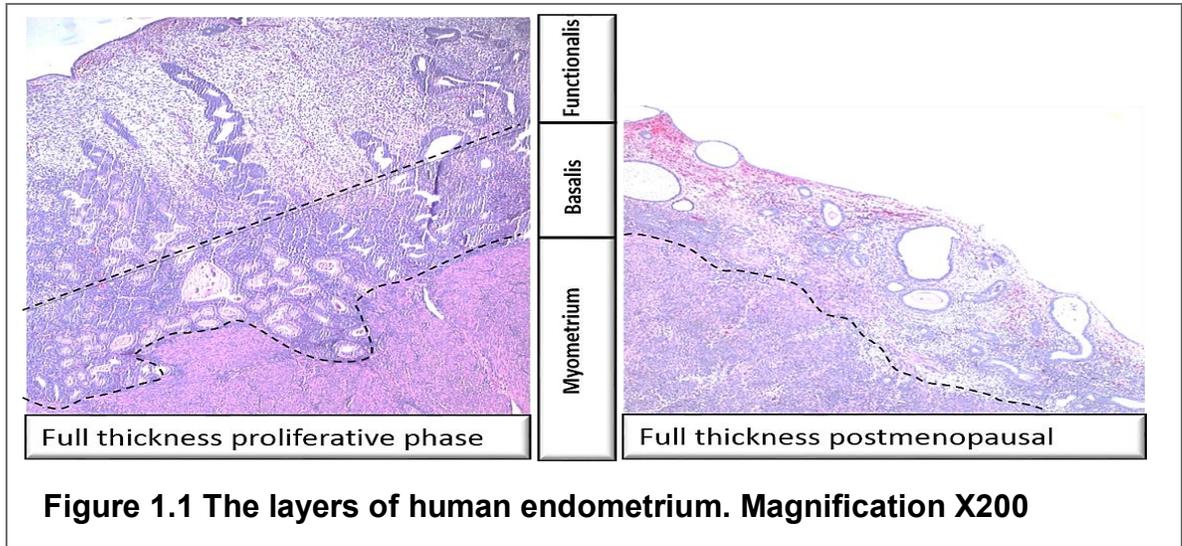
1.1 Endometrial ontogeny

1.1.1 Embryology

In all mammals including humans, the uterus develops when the embryonic Mullerian ducts fuse in the midline with the inner mesodermal layer forming the endometrium (Gray et al., 2001). Initially, the superficial endometrial glands originate from the foetal undifferentiated single columnar epithelial cells *in utero*. The histological architecture of the endometrium at birth is shallow and superficial (Ellenson et al., 2011).

1.1.2 Normal endometrium

By puberty, the endometrium acquires a mature architecture. Unlike other mammals, humans (and other upper order primates) menstruate, and have two distinct layers of endometrium (Figure 1.1). The transient superficial stratum functionalis exists only during the reproductive life of women whilst the structurally permanent deeper stratum basalis adjacent to the myometrium, is thought to give rise to the functionalis and remains throughout life (Hapangama et al., 2015).



The endometrium consists of a variety of cell types including epithelial, stromal, endothelial and leukocyte populations. The endometrial epithelium can be further subdivided to three distinct groups of cells:

1. Luminal epithelium: lines the superficial surface of the uterine cavity.
2. Functionalis glandular epithelium: fully differentiated, hormone responsive epithelium just under the luminal epithelium.
3. Basalis glandular epithelium: the deeper SSEA-1 expressing epithelial cells adjacent to the myometrium (Valentijn et al., 2013).

A new functionalis layer is presumed to be generated from the remaining basalis after menstrual shedding; hence, the basalis is expected to harbour stem/progenitor cells (Valentijn et al., 2013).

1.2 Endometrial hyperplasia

Endometrial hyperplasia (EH) is a pathologically heterogeneous diagnosis that ranges from histologically subtle and spontaneously reversible proliferative lesions to premalignant changes (Montgomery et al., 2004; Mazur, 2005). It virtually always results from prolonged oestrogen stimulation, unopposed by

progesterone. EH without cytological atypia occurs in the early postmenopausal years (50-54 years) whereas the incidence of those with cytological atypia (EHA) peaks in the early sixties (Reed et al., 2009). EHA is associated with a significantly increased risk of EC (RR=14, 95% CI, 5–38) and is recognised as the precursor of the endometrioid type of EC (Lacey et al. 2007).

All forms of EH share certain morphological features, showing an increase in the gland to stroma ratio, irregularities in the shape and size of the glands (Robert J. Kurman Brigitte M. Ronnett, 2011). At a cellular level, the change in the cytology of EH has been recognised as the principal indicator for malignant transformation susceptibility. The previous WHO classification (1994) divided EH into discrete categories; *simple* and *complex, with or without cytological atypia*. This intricate classification has resulted in a considerable confusion and inconsistency in reporting and subsequent management (Emons et al., 2015). Increasing evidence indicates that architectural classification of EH to *simple* and *complex* has limited value in predicting outcome; therefore, WHO implemented a new simplified classification based entirely on the cytological changes of glandular epithelium (Table 1.1), thus dividing EH into *EH without cytological atypia* and *atypical EH* (Zaino et al., 2014).

Hyperplasia without atypia is a reversible benign change, which regresses after alteration of the endocrine milieu, for example, with progesterone treatment. Progression to invasive disease occurs only in 1–3% if unopposed oestrogen persists for a long term (Emons et al., 2015). Importantly, these lesions exhibit no relevant genetic changes. By contrast, atypical endometrial hyperplasia exhibits mutations typical for invasive endometrioid endometrial cancer (Kandoth et al., 2013). In up to 60% of cases, either patients have coexisting invasive cancer or they are at extremely high risk of subsequently developing invasive cancer (Antonsen et al., 2012).

Table 1.1 The new classification of endometrial hyperplasia, table adapted from Emon et. al. (2015).

New classification	Previous equivalent terms	Molecular changes	Endometrial cancer Risk ratio
Hyperplasia without atypia	<ul style="list-style-type: none"> • Simple hyperplasia no atypia • Complex hyperplasia no atypia 	Few somatic mutations in scattered glands	RR: 1.01–1.03
Atypical hyperplasia	<ul style="list-style-type: none"> • Complex atypical hyperplasia • Simple atypical hyperplasia 	<ul style="list-style-type: none"> • Micro satellite instability • PAX2 inactivation • Mutation of PTEN, KRAS and CTNNB1 	RR: 14–45

Endometrial intraepithelial carcinoma (EIC) is the precursor lesion of serous carcinoma and is completely different from atypical hyperplasia. EIC represents a non-invasive glandular lesion characterised by epithelial cells with marked nuclear abnormalities resembling those seen in serous EC. Unlike EH with atypia, EIC is not typically associated with increased oestrogen exposure. By contrast, it is seen in the atrophic endometrium of older postmenopausal women (Horn et al., 2007).

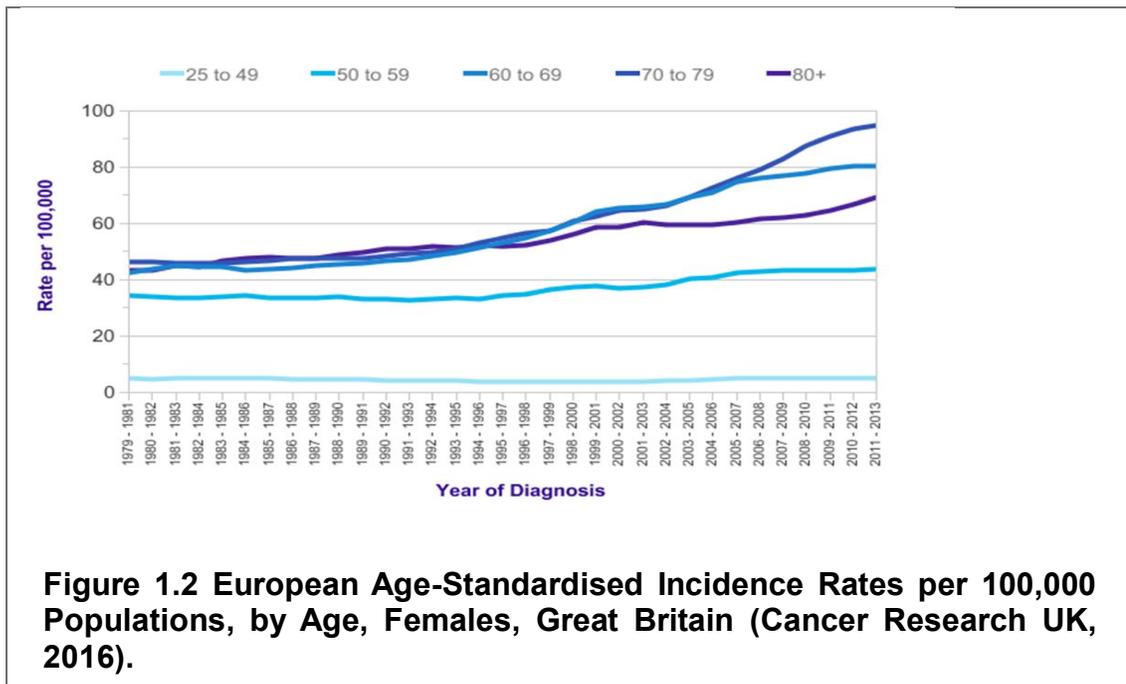
1.3 Endometrial cancer

1.3.1 Epidemiology

EC is the most common female genital tract cancer in the Western World. It is the sixth most common cancer in women worldwide and the fourteenth most common cancer overall. The highest incidence is found in North America, with age-standardised incidence rates of approximately 18 per 100,000 in US whites and 15 per 100,000 in Canadians (Ferlay et al., 2015). Age-standardised incidence rates are also particularly high in Europe, especially Eastern Europe in countries

such as the Czech Republic, Slovakia, and Latvia, where rates are comparable to those in US Whites (Komodoki, 2006). In the UK, which is estimated to be the 20th highest in Europe, EC accounts for 5% of all new cases of cancers in females with the highest age-standardised incidence rates recorded in Wales reaching 28 per 100,000 (Cancer research UK, 2016). The incidence rates of ECs are generally low in southern and eastern Asian including Japanese, and in most Africans and middle eastern Arabs which estimated to be less than 6 per 100,000 (Komodoki, 2006).

In contrast to breast and cervical cancers that are expected to decline significantly by 2030, EC is expected to increase over the next decade (Mistry et al. 2011). The largest increase overall, so far, has been seen in women aged 65-79 years, however, increases in younger age groups (25-49 years and 50-59 years) are equally as alarming, Figure 1.2.(Cancer research UK, 2016).



EC is the 9th most common cause of cancer death among women in the UK (2015) and the 14th most common cause of female cancer death worldwide.

Uterine cancer mortality bears a strong correlation with age, with 49% of mortality rates recorded in women aged 75 years and over (Cancer research UK, 2016).

1.3.2 Risk Factors

The risk of women to develop EC depends on many factors; with lifestyle factors linked to 37% of cases (Cancer research UK, 2016). Risk factors for EC are associated with lifetime exposure to oestrogen and can be categorised in the following way:

1.3.2.1 Factors associated with higher EC risk

1. **Age:** More than 90% of EC cases occur in women older than 50 years of age, with a median age of 63 years (Colombo et al. 2013) and the risk increasing with advancing age. For example, a woman under the age of 40 years has a 1 in 1,423 risk of developing the disease, whereas a woman older than 70 years has a 1 in 81 risk (Siegel et al., 2012).
2. **Race:** Cancer research UK does not include race as a risk factor; however, several studies have indicated that white Americans have a 12% higher lifetime risk of EC compared with African Americans (Howlader et al., 2015). Paradoxically, African American mortality rate is reported to be 86% higher if they develop EC. Studies have shown higher grades and more aggressive tumours in African-Americans than in other ethnic groups even when comparing patients with the same stage at diagnosis (Sherman and Devesa, 2003; Setiawan et al., 2007). A recent study concluded that cancer-specific deaths were similar in both groups and the increase in overall mortality was due to other causes (Rauh-Hain et al., 2015). Socioeconomic status and the type of health care insurance were investigated as a cause of the disparity in mortality rate between these two groups. Dolly and colleagues have shown that mortality rate was significantly associated with a longer interval of time from diagnosis to surgery for both races regardless of the type of health insurance (Dolly et al., 2016).

3. **Obesity:** Over one-third of ECs occurring in the UK each year are associated with obesity (Parkin et al., 2011). Meta-analytic studies have shown that the risk of EC increases by 81% per 5Kg of weight gain. Both overweight and obese women are at higher risk of developing EC, with the risk doubling in the latter group compared with a healthy weight cohort, BMI<25 (Crosbie et al., 2010; Y. Zhang et al., 2013). Body fat has been proposed as a cause of EC and linked to: a) ability of adipose tissue to convert androgens to oestradiol (Ali, 2014) , b) hyperinsulinemia and insulin resistance (Esposito et al., 2014) and c) downregulation of adipokines (Luhn et al., 2013).
4. **Increased number of menstrual cycles:** The increase in the number of years between menarche and menopause excluding pregnancy has been associated with increased risk of EC (McPherson et al., 1996). A higher incidence of EC was reported in women who has reached menarche at 12 years of age or earlier (Brinton et al. 1992; Ali 2014). Conversely, those who undergo menopause at 52 years of age or later have twice the risk of developing EC as women who undergo menopause under the age of 50 years (Zucchetto et al., 2009).
5. **Polycystic ovarian syndrome (PCOS):** When a woman has PCOS her risk of developing EC is 3.8 times higher than a woman without the condition. This increases to a risk factor that is 4.1 times higher in premenopausal women (aged ≤54 years) (Barry et al., 2014). Several mechanisms have been suggested to be involved alongside unopposed oestrogen exposure; the most of which being insulin resistance (Palomba et al., 2009).
6. **Endometrial polyps:** Endometrial hyperplasia and cancer have been observed in 5.42% of endometrial polyps from PM women compared with 1.7% in reproductive age women (Lee et al., 2010). Oestradiol (E2) and to lesser extent tamoxifen are proposed as the driving force behind endometrial polyp formation however direct functional evidence for malignant transformation is lacking (Van Bogaert, 1988; Erdemoglu et al., 2008).

7. **Hormone replacement therapy (HRT):** The association of PM HRT with increased EC risk is well established. In the UK, an estimated 1% of uterine cancers each year are linked to HRT use (Parkin, 2011). Compelling studies have shown that oestrogen-only HRT increases EC risk by 2.3 times (Grady et al., 1995); although a limited additional impact of exogenous oestrogen has been observed in overweight and obese women probably as a result of high endogenous oestrogen (Crosbie et al., 2010). Low dose oestrogen has not been associated with increased EC risk, but a randomised clinical trial has shown an increase in EH risk after 18-24 months of treatment (Furness et al., 2012). EC risk decreases after cessation of oestrogen-only HRT use, though the risk was still higher in ex-users than never users at least five years after cessation (Grady et al., 1995). On the other hand, accumulating evidence has indicated that continuous use of combined HRT reduces EC risk by 22% (Furness et al., 2012; Brinton and Felix, 2014). Increased risk of EH has not been shown with continuous or sequential combined HRT (Brinton and Felix, 2014).
8. **Tamoxifen:** The International agency for research on cancer classified tamoxifen as a cause of EC. EC risk among breast cancer survivors is 3 times higher in those aged 55-69 years who used tamoxifen for approximately 5 years (Cohen, 2004).
9. **Previous cancer:** It has been suggested that EC risk is higher in breast and ovarian cancer survivors (Jégu et al., 2014) perhaps because they share the same lifestyle and environmental factors, for example, excess body weight (Druesne-Pecollo et al., 2012).
10. **Diabetes:** The risk of EC is 40-81% higher in diabetic patients compared with non-diabetics which may largely be associated with excess body weight and insulin resistance (Starup-Linde et al., 2013; Z.-H. Zhang et al., 2013; Luo et al., 2014).
11. **Parkinson's disease (PD):** Several epidemiological studies have associated PD with a decreased risk of cancer (Minami et al., 2000; Driver et al., 2007).

Conversely, a recent pooled cohort study of 219,194 patients has reported a significantly increased EC risk (RR,1.17) in PD patients, although this significance has been lost when correction for multiple comparisons was applied (Ong et al., 2014). With the knowledge that PD associated hormonal risk factors are opposing the EC risk factors, such as lower number of menstrual cycles and inverse association with oestrogen (Bourque et al., 2009), the association of PD with increase EC risk is not expected, and clarification of this query warrants further studies.

12. **Others:** Limited evidence has suggested that patient's height and red meat consumption could be possible risk factors for EC. A recent pooled study of 38,862 participants has reported an association between height per 5cm increment and overall female cancer risk and mortality, yet association with EC was not significant (Green et al., 2011; Wirén et al., 2014). Other studies have suggested an association between 100g/day red meat consumption and EC risk increase; RR 1.5. (Bandera et al., 2007a) due to several factors such as food processing chemicals and oestrogen content of these products.

1.3.2.2 Factors associated with lower EC risk

1. **Contraceptives:** The protective effect of combined oral contraceptives (COCP) against EC has been well- documented (Collaborative Group, 2007) . A meta-analytic study has reported recently showing a stepwise increase in EC risk reduction and the duration of COCP consumption, every 5 years of use was associated with a relative risk reduction of 0.76 (Setiawan et al., 2013; Collaborative Group on Endometrial Epidemiological Studies on cancer, 2015). This lowering of risk is shown to be limited to women with over 5 years of COCP use, yet the persistent favourable outcome may continue up to 30 years after cessation of COCPs (Cook et al., 2014). These findings were consistent with both endometrioid and nonendometrioid subtypes (Setiawan et al., 2013) with carcinosarcoma being the exception and reports conflicting between weak and no association (Felix et al., 2013). Limited evidence exists for a similar protective role with the progesterone only pill (POP) users. The long

term protective role of levonorgestrel-releasing intrauterine system (LNG-IUS) against EC is not clear, although several studies have reported effectiveness for the treatment of EH (Mueck et al., 2010).

2. **Parity:** A decrease in EC risk has been noticed in women with a greater number of full term pregnancies. Age of the women at the last pregnancy has been proportionally associated with a reduced EC risk. Women who last gave birth at 40 years of age or older had a 44% decreased risk compared with women who had their last birth under the age of 25 years (Setiawan et al., 2012). This has been explained by increased progestogen levels and certain physical changes to the uterus during and after childbirth. Compared to parous women, a 35-42% higher risk has been associated with nulliparous women.
3. **Smoking:** The inverse relationship between cigarette smoking and EC risk is well established (Cancer research UK, 2016). According to a recent study, reduced EC risk was evident among former (RR 0.89, 95 % CI 0.80, 1.00) and current (RR 0.65, 95 % CI 0.55, 0.78) smokers compared with those who had no previous history of being a smoker (Felix et al., 2014). This protective effect is expected to be via interfering with the endocrine glands function including the adrenals and ovaries; resulting in steroid hormone modulation. Anti-oestrogenic mechanisms have been suggested as the prominent effector through increasing the hepatic metabolism of oestrogen into minimally active 2-hydroxyestrogens (Michnovicz et al., 1986), shortening of the follicular phase and inducing early menopause (Mattison and Thorgeirsson, 1978; Spangler, 1999). However, several studies have also indicated that adrenal androgen production in smoker women was higher than non-smokers (Brand et al., 2011) and associated with higher circulating androstenedione and testosterone levels (Kapoor and Jones, 2005; Brand et al., 2011).
4. **Others:** Lifestyle and nutritional studies have suggested several factors are associated with lower EC risk based on systematic reviews, pooled or meta-analyses. Of these, physical activity (Keum et al., 2014), cruciferous

vegetables (Bandera et al., 2007b), coffee (Je and Giovannucci, 2012) and tea (Tang et al., 2009) have been shown to reduce the risk of EC.

1.3.2.3 Factors which have no association with EC risk

Meta-analysis, pooled analysis or systematic reviews have shown that the following factors have no association with EC risk (Cancer research UK, 2016):

1. Ovarian hyperstimulation for *in vitro* fertilisation (Siristatidis et al., 2013).
2. Diethylstilbestrol exposure: Prenatal diethylstilbestrol exposure is associated with excess risks of clear cell adenocarcinoma of the vagina and cervix in young women (Sassoon, 1999; Troisi et al., 2007) and breast cancer in older women. Studies have reported that EC risk is not associated with DES exposure in pregnancy or in utero (Titus-Ernstoff et al., 2001; Troisi et al., 2007).
3. Human papillomavirus (Olesen et al., 2014).
4. Alcohol (Friberg et al., 2010).
5. Statins (Liu et al., 2014).
6. Fish, poultry, dairy, fruit and vegetable consumption (Bandera et al., 2007a, 2007b; Stevens et al., 2014).
7. Vitamin D blood levels (Skaaby et al., 2014) or supplement (Bjelakovic et al., 2014).

1.3.3 Classification of endometrial cancer

EC comprises a biologically, clinically, morphologically, and genetically heterogeneous group of tumours. Over the last decade, several classifications have been proposed aiming at improving prognosis and predicting function. The basis and characteristics of each classification system are summarised in this section:

1.3.3.1 WHO

Based on the origin of the neoplastic cell and histological morphology the WHO classified EC into 7 categories (Zaino et al., 2014):

1. Epithelial tumours (Table 1.2)
2. Mesenchymal tumours; eg, endometrial stromal and smooth muscle tumours.
3. Mixed epithelial and mesenchymal tumours; eg, carcinosarcomas.
4. Gestational trophoblastic diseases and other malignant tumours.
5. Miscellaneous tumours; eg neuroectodermal tumours.
6. Lymphoid and myeloid tumours; eg Lymphomas.
7. Secondary tumours.

This classification limitation is that carcinosarcoma was considered as a mixed epithelial mesenchymal tumour whereas research has shown these biphasic tumours to be clonally derived from a transformed epithelial cell (Ellenson et al., 2011). Hence, alternative classifications were proposed to overcome these limitations.

Table 1.2 WHO classification of epithelial endometrial cancer (Zaino et al., 2014).

WHO classification of epithelial endometrial cancer
Endometrioid adenocarcinoma <ul style="list-style-type: none"> ▪ <i>Villoglandular</i> ▪ <i>Secretory</i> ▪ <i>Ciliated cell</i> ▪ <i>Endometrioid adenocarcinoma with squamous differentiation</i>
Serous carcinoma
Clear cell carcinoma
Mucinous carcinoma
Neuroendocrine tumours <ul style="list-style-type: none"> • Low grade (carcinoid) • High-grade small cell neuroendocrine carcinoma; large cell neuroendocrine carcinoma
Squamous carcinoma
Mixed types of carcinoma
Undifferentiated carcinoma
De-differnetiated

1.3.3.2 Dualistic

Three decades ago, clinical, metabolic and epidemiological features of EC were incorporated to generate the widely accepted dualist model (Bokhman, 1983), which was further supported by molecular profile characteristics for each proposed subtype (Sherman, 2000), Table 1.2. This classification broadly divided EC into two major categories:

1. Type I cancers; represent approximately 80% of ECs and arise in relatively younger peri and PM women. Typically, cancers in this category are of low grade endometrioid subtype with indolent clinical behaviour arising on a hyperplastic background and they are associated with signs of unopposed oestrogens, hyperlipidemia, and obesity.
2. Type II cancers; includes serous, clear cell and carcinosarcoma which usually arise on an atrophic endometrium background of older PM women and generally have an aggressive clinical course. Cancers of this category are

thought to be hormonally independent; however, recent evidence has shown common risk factors between the two subtypes (Setiawan et al., 2013).

A major limitation of this classification is that it failed to define some of the high grade, type I, endometrioid EC which have molecular aberration patterns and clinical outcomes that blends between type I and type II ECs (Voss et al., 2012).

1.3.3.3 Molecular

The recent emergence of a large cohort of endometrioid, serous and mixed EC genotype and the subsequent reports of integrated proteomic and transcriptomic profiles of them has added a new perspective to EC taxonomy (Murali et al., 2014). The Cancer Genome Atlas (TCGA) study identified four genomic groups (Table 1.3);

1. POLE ultramutated: this group is characterised by a stable somatic copy number albeit highly mutant genes particularly POLE; a catalytic subunit of DNA polymerase epsilon involved in nuclear DNA replication and repair. The hallmark of this group is favourable outcomes even with high grade tumours (Meng et al., 2014; Billingsley et al., 2015; Church et al., 2015).
2. Microsatellite instability (MSI): consists mainly of the endometrioid subtype and has mutations tenfold higher than copy number low, with less favourable outcomes.
3. Copy-number low: similar to MSI group, consists mainly of endometrioid EC. It is characterised by 100% mutation of chromosome 1 and 52% mutation in Beta catenin (CNNB1).
4. Copy-number high: consists mainly of serous EC and 24% G3 endometrioid. Characterised by TP53 mutation, FBXW7 and PPP2R1A amplification with an exceptionally poor outcome.

This classification is in harmony with the dualistic model, POLE ultramutated, microsatellite instability and copy number low groups comprise 97% of type I EC

while the copy number high group encompasses 94% serous EC; nonetheless, 24% of grade 3 endometrioid are also resolved to the worst outcome high copy number group confirming the previously predicted overlap between the subtypes (Kandoth et al., 2013). This discrepancy warrants integration of clinic-pathological, hormonal, molecular and genomic parameters and a more holistic approach in the future to improve postsurgical stratification and tailoring personalised adjuvant therapy for women with EC in order to improve outcomes (Talhouk et al., 2015).

1.3.3.4 Modified dualistic

Taking into consideration the molecular signature and clinical behaviour of grade 3 endometrioid EC, it is conceivable to modify the dualistic model so that grade 1 and grade 2 endometrioid are grouped as low grade EC (LGEC) and grade 3 endometrioid is added to type II EC which are histologically high grade EC (HGEC) and associated with common molecular changes and unfavourable outcomes as shown in Table 1.3. This classification is being increasingly used in EC research and incorporation in the FIGO staging system has been suggested, to reduce heterogeneity of stage I and to improve outcome prediction (Barlin et al., 2013).

Table 1.3 Classification systems for endometrial cancer, table adapted from Morice et. al. (2015).

	Basis	Categories	Histological type	Tumour grade	Molecular changes
WHO	Historical features	Epithelial	Endometrioid Serous Clear cells Undifferentiated Dedifferentiated Neuroendocrine	Grade 1-3 Grade 3 Grade 3 Grade 3 Grade 3 Low / high	
		Mixed epithelial/mesenchymal	Carcinosarcoma	Grade 3	
		Mesenchymal			
		Gestational, miscellaneous Lymphoid secondary			
Dualistic	Clinical and epidemiological features	Type I	Endometrioid	Grade 1-3	PTEN (52–78%, PIK3CA (36–52%), PIK3R1 (21–43%), KRAS (15–43%), ARID1A (25–48%), CTNNB1 (23–24%), MSI (28–40%).
		Type II	Serous, clear cells and carcinosarcoma	Grade 3	TP53 (60-91%), PPP2R1A (15-34%), HER2 (27,44%)
Molecular	Genome-wide genomic characterisation	POLE ultramutated	Endometrioid	Grade 1-3	POLE (100%), PTEN (94%), PIK3CA (71%), PIK3R1(65%), FBXW7 (82%), ARID1A (76%), KRAS (53%), ARID5B (47%), MSI variable
		MSI hypermutated	Endometrioid	Grade 1-3	PTEN (88%), RPL22 (37%), KRAS (35%), PIK3CA (54%), PIK3R1 (40%), ARID1A(37%), MSI high
		copy-number low	Endometrioid	Grade 1,2	PTEN (77%), CTNNB1 (52%), PIK3CA (53%) PIK3R1 (33%), ARID1A (42%), MSI stable
		copy-number high	Serous, endometrioid, mixed serous and endometrioid	Grade3	TP53 (92%), PPP2R1A(22%), PIK3CA (47%) MSI stable
Modified dualistic	Re-categorizing grade 3 endometrioid	Low grade EC	Endometrioid	Grade 1-2	
		High grade EC	Endometrioid, serous, clear cell, carcinosarcoma	Grade 3	

1.3.4 Histological types of endometrial cancer

1.3.4.1 Endometrioid EC

Endometrioid subtype accounts for at least 85% of EC cases. They resemble proliferative phase endometrium for which they were given this name. Microscopically, transformed glands form a confluent pattern, uninterrupted by stroma in the form of the cribriform pattern, extensive papillary pattern or an irregular infiltration of glands associated with an altered fibroblastic stroma (Ellenson et al., 2011; Buhtoiarova et al., 2016).

The grade of endometrioid carcinoma is determined by the histological appearance/differentiation, detected by microscopy. The FIGO grading system, most commonly used for grading endometrioid and mucinous carcinomas, is based upon the extent of non-gland forming tumour combined with an assessment of the nuclear morphology (Table 1.4). Serous, clear cell, undifferentiated carcinoma, and carcinosarcoma are not graded and are, by definition, regarded as high grade (grade 3) carcinomas (Ellenson et al., 2011).

Table 1.4 FIGO grading system for endometrial cancer (Ellenson et al., 2011).

	Architectural	Nuclear
Grade 1	Less than 5% nonsquamous, or nonmorular solid growth pattern.	Oval/elongated nuclei, fine chromatin, small nucleoli, few mitoses
Grade 2	6% to 50% nonsquamous, or nonmorular solid growth pattern.	Features between grades 1 and 3
Grade 3	More than 50% nonsquamous, or nonmorular solid growth pattern.	Enlarged/pleomorphic nuclei, coarse chromatin, prominent nucleoli, many mitoses

1.3.4.2 Serous EC

Serous subtype is a high grade EC, accounting for less than 10% of all EC. Although a papillary pattern typically predominates, glandular and solid patterns also occur. Cells are partly discohesive polygonal shape with high nuclear-to-cytoplasmic ratios and a high mitotic index. Characteristically, marked (high grade) nuclear atypia is required for a tumour to qualify as serous carcinoma. Cell detachment, budding, and tufting are usually present, typically resulting in luminal borders that appear ruffled, in contrast to the linear, smooth contours of endometrioid carcinoma (Soslow et al., 2007). The spread of serous carcinoma is commonly intra-abdominal, in a manner resembling ovarian cancer. Intra-abdominal metastases occur early and are often present at diagnosis in the absence of myometrial invasion (Ellenson et al., 2011).

1.3.4.3 Clear cell EC

Endometrial clear cell carcinomas are rare and constitute 1% - 6% of all EC (Ellenson et al., 2011). They show papillary, glandular, tubule-cystic, and/or solid architecture. The predominant cell type lining glands and the papillae are hobnail or cuboidal. Solid areas may have a cobblestone appearance, composed of polygonal, clear cells due to glycogen, with sharp cytoplasmic borders and a nucleolus. The stroma in clear cell carcinoma is distinctive, has inflammation, and is hyalinised and myxoid. Clear cell carcinomas usually have a mitotic index that is lower than that of serous carcinoma (Ellenson et al., 2011; Soslow, 2013)

1.3.4.4 Carcinosarcoma (CS)

Immunohistochemical and molecular genetic studies support the clonal origin of both tumour components (epithelial and mesenchymal-like elements) and are now thought to be “undifferentiated” or “metaplastic” carcinomas rather than uterine sarcomas. CS account for 2% to 5% of all EC (Ellenson et al., 2011). The epithelial element usually has features of high-grade endometrial adenocarcinoma, often serous or clear cell carcinoma, although endometrioid

patterns, including carcinoma with squamous differentiation, may be found. The sarcomatous component may be homologous or heterologous. The homologous sarcoma-like component often contains hypercellular sheets of small, hyperchromatic, round to spindle-shaped cells with a high mitotic rate and they lack apparent differentiation. The prognosis is usually determined by the epithelial component, and this component is present in most metastases, including foci of vascular invasion. Patients with a non-endometrioid epithelial component have unfavourable outcomes in comparison to those with an endometrioid epithelial component (Ellenson et al., 2011; Soslow, 2013).

1.3.4.5 Mixed tumours

The endometrioid tumour may show foci of serous or clear cell types. When high grade subtype constitutes at least 10% of the tumour it is termed a mixed tumour. Mixed endometrioid tumour with a serous or clear component equal or more than 25% behaves as pure serous or clear carcinoma respectively (Ellenson et al., 2011).

1.3.5 Pathogenesis of endometrial cancer

Like all other tumours, ECs develop when the endometrium acquires six biological capabilities/hallmarks, in a multistep pattern (Figure 1.3). These hallmarks include; persistent proliferation signalling, evasion of growth suppressor, resistance to cell death, cell immortality, induction of angiogenesis and activation of invasion and migration pathways (Hanahan and Weinberg, 2011). Currently, science is not able to explicitly explain endometrial carcinogenesis; nonetheless, three main signalling pathways have been identified to be consistently involved (Markowska et al., 2014).

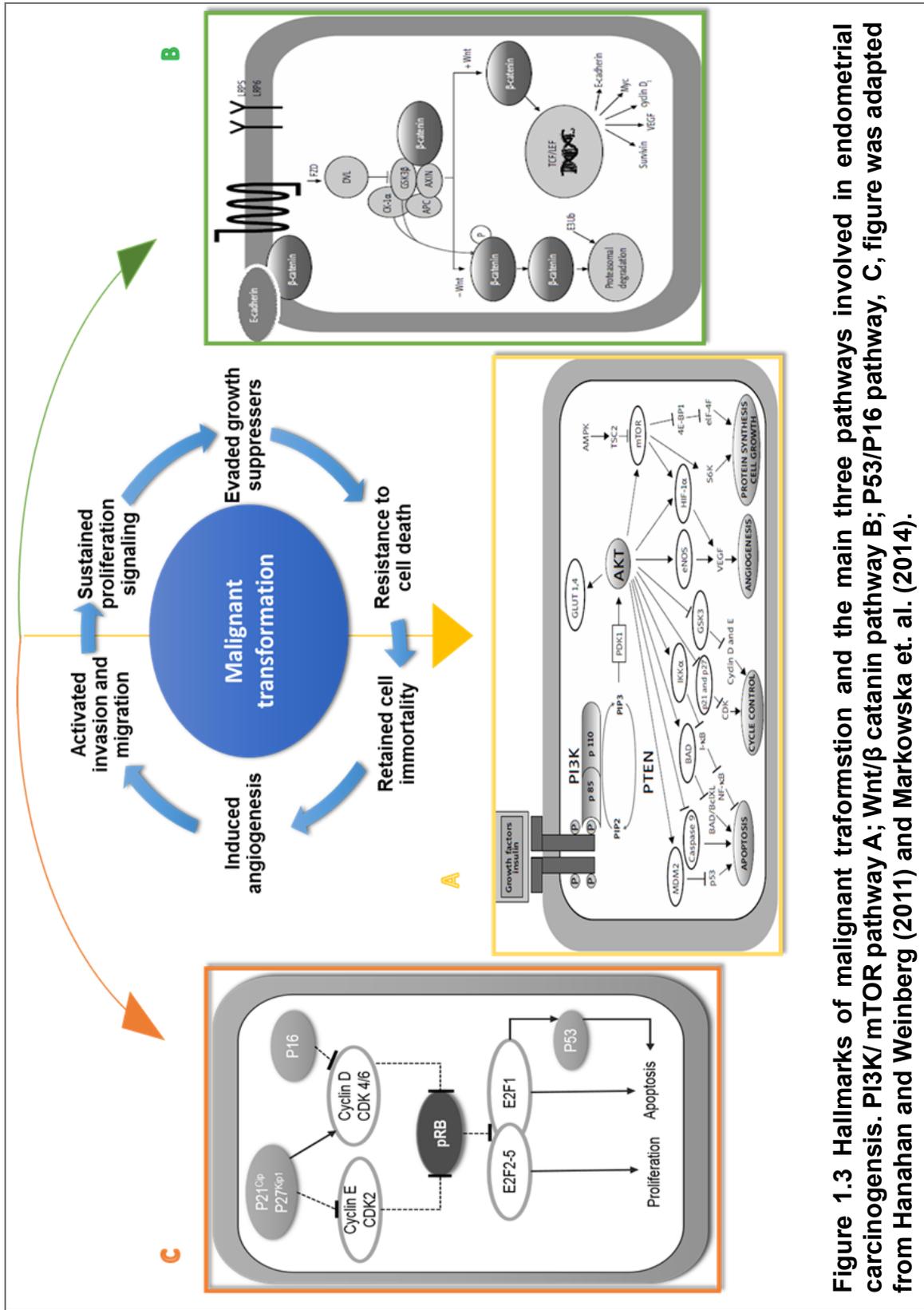


Figure 1.3 Hallmarks of malignant transformation and the main three pathways involved in endometrial carcinogenesis. PI3K/mTOR pathway A; Wnt/β catenin pathway B; P53/P16 pathway, C, figure was adapted from Hanahan and Weinberg (2011) and Markowska et. al. (2014).

1. PI3K/ Akt/ mTOR pathway: The PI3K/Akt pathway is a key regulator of survival during cellular stress (Datta et al., 1999). Uncontrolled PI3K receptor activation with subsequent unrestrained proliferation is one of the best recognised pathways in EC (Matias-Guiu and Prat, 2013). Through the PI3K-Akt, different cellular stimuli such as insulin-like growth factor-1 (IGF-1), members of the human epidermal growth factor receptor family, and vascular endothelial growth factor receptors (VEGFRs) transmit signals to the mammalian target of rapamycin (mTOR) to regulate downstream signalling and protein synthesis (Figure 1.3A). Hypermethylation of phosphatase and tensin homolog (PTEN), a counter regulator in AKT phosphorylation, leads to further over activation of mTOR. (Porta et al., 2014). Amplification of P13KCA genes have been observed simultaneously in 46% vs. 24% of cancers with normal PTEN (Oda et al., 2005)..
2. Wnt/ β -catenin pathway: Typically, the canonical Wnt pathway promotes cell proliferation, controls fate determination and terminal differentiation (Clevers et al., 2006). Wnt signalling is involved in regulating progenitor cell populations in diverse tissues and remains essential throughout life. The first link between human cancer and Wnt/ β -catenin was demonstrated via the interaction between β -catenin and APC (Rubinfeld et al., 1993). Abnormal activation of the Wnt pathway has been identified in around 40% of ECs mainly endometrioid. As shown in Figure 1.3B target genes expression (Myc, cyclin D, VEGF, E-cadherin, survivin) are promoted either by activation of the canonical pathway or by mutation of its key genes CNNB1, APC or axin. These genes subsequently drive cell proliferation, inhibit apoptosis or promote detachment (Polakis et al., 2000).
3. Cell cycle regulators: Deregulation of the cell cycle machinery results in abnormal cell growth. P53 is an important cell cycle checkpoint and a suppressor protein which can arrest growth by holding the cell cycle at the G1/S regulation point on DNA damage recognition.

p53 mutation more often affects nonendometrioid cancers, 93–100%, particularly serous carcinomas (Ragni et al., 2005). The interplay between p53 and IGF-I signalling pathways have been recently described, p53 has been involved in IGF-IR gene expression regulation via Sp1 transactivator protein (Attias-Geva et al., 2012). Another cell cycle regulator which prohibits cell progression from G1 to S by inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate pRb (Serrano et al., 1993) is p16, Figure 1.3C. Aberrations in this pathway have been seen in 78% of serous carcinomas and 36% of endometrioid cancers (Netzer et al., 2011). As a consequence of p16 inactivation, Rb overexpression pushes the endometrial cell from G1 to S and induces proliferation (Tsuda et al., 2000).

Disruptions of these three pathways fuel neoplastic transformation, initiated by accumulation of chromosomal aberrations. Furthermore, hormonal and inflammatory factors strongly attribute to the activation of these pathways in EC which ultimately lead to uncontrolled cell growth, invasion, and metastasis.

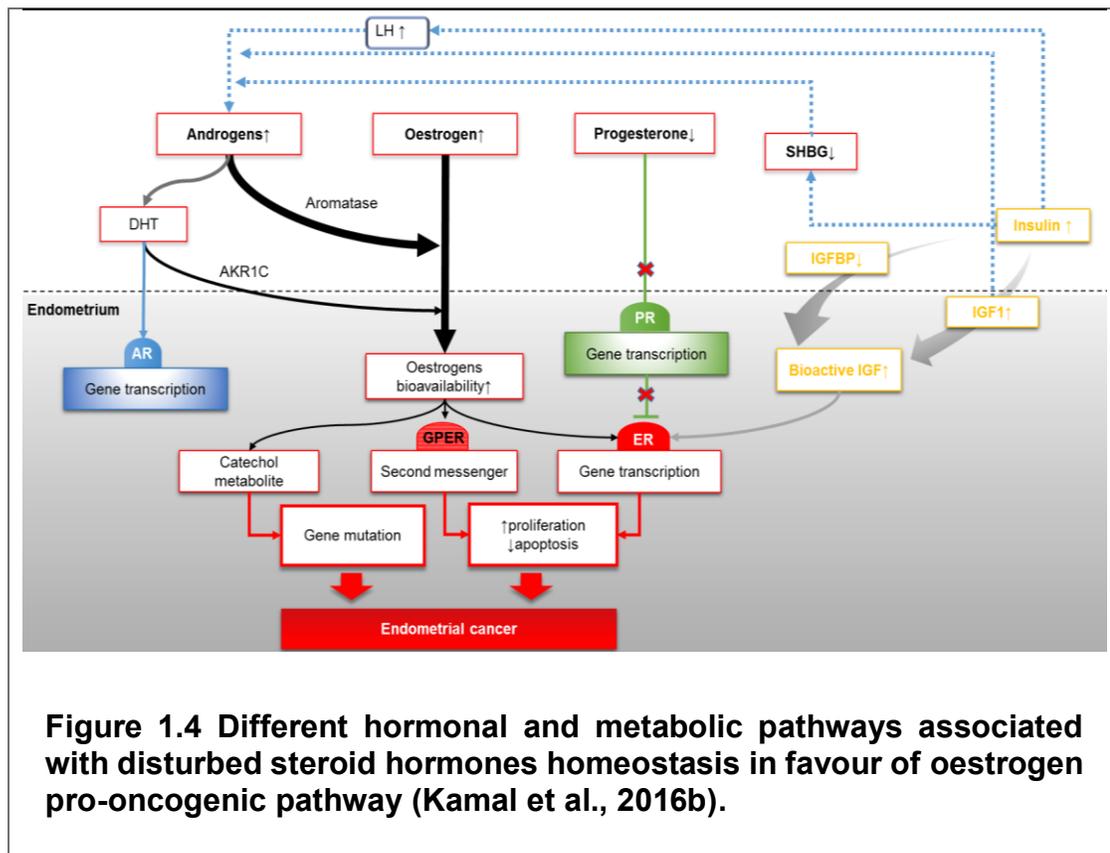
1.3.6 Sex steroid hormones and endometrial carcinogenesis

The endometrium is the target for ovarian hormones. Prolonged unopposed oestrogen exposure has been strongly linked to increased EC risk as mentioned previously. This section covers the available evidence of the roles that steroid hormones play in endometrial carcinogenesis (Figure 1.4).

1.3.6.1 Oestrogen

Oestrogen is the primary female sex hormone responsible for the development and regulation of female reproduction function and secondary sexual characteristics. Normally, in non-pregnant women, circulating oestrogens are produced mainly by granulosa cells of the ovary under the influence of follicular stimulating hormone (FSH). A smaller amount is produced by extragonadal organs such as the liver, adrenals, breasts and fat. There are three major

endogenous oestrogens; the first and most potent is oestradiol (E2) which predominates in reproductive life, the second is oestrone (E1), a weak oestrogen that predominates after menopause and the third is oestriol (E3), the least potent of the three, which is produced by the placenta.



The basic impact of oestrogens on reproductive tissue proliferation and growth was recognised in the 1950s (Jensen and DeSombre, 1973), 10 years before identification of oestrogen receptors (Toft and Gorski, 1966). Following that, research has been focused on investigating the contribution of oestrogen in neoplastic transformation of hormone dependent malignancies mainly breast cancer, and to a lesser extent EC. Although not completely decoded, several pathways have been proposed involving molecular, biological and chemical mechanisms:

1. **Genetic pathway:** is the best characterised and well accepted pathway, oestrogen responsive gene transcription regulates proliferation, growth,

differentiation, angiogenesis and apoptosis inhibition. Transcription of oestrogen responsive genes can be induced by:

- *Classical oestrogen response element (ERE) dependent pathway*; oestrogen activated ER (α or β), dimerises and translocates to the nucleus, binds DNA at the promoter of the responsive gene elements (ERE) and activates the expression of ERE-dependent genes, after recruiting coactivators (O'Malley, 2005).
 - *Non-classical, ERE-independent, pathway*: this involves activation of membranous/cytoplasmic ER forms which in turn activate DNA binding of transcriptional factors in a so called protein-protein interaction, such as SP-1 (O'Lone et al., 2004), IGF-1 receptor (Umayahara et al., 1994), EGF receptor (Pietras and Márquez-Garbán, 2007), PA-1 (Kushner et al., 2000) and NF- κ B (Ray et al., 1994).
 - *Ligand-independent pathway*: both nuclear and cytoplasmic ER α transcriptional activity via co-regulator phosphorylation can be induced by growth factors (Kato et al., 1995). In an ovariectomised murine model, treatment with EGF or IGF-I resulted in an increase in uterine weight and proliferation of the uterine epithelial cells. Importantly, these effects were not observed in ER α knockout mice, suggesting that ER α is required for growth factor action (Hewitt et al., 2005).
2. **Non genetic pathway**; a quick, non-transcriptional response to E2 via membrane located ER has been described. Activated receptors initiate calcium (Ca^{2+}) influx which consequently activates Erk1/2 signalling pathway (Zhang et al., 2009). Membrane receptors, unrelated to conventional steroid receptors, have also been implicated in this pathway. GPR30, an orphan member of the G protein-coupled receptor superfamily, has been shown to bind oestrogen (Thomas et al., 2005) and stimulate EC cell proliferation and invasion by increasing the production and activity of MMP-2 and MMP-9 via the MEK/ERK MAPK pathway (Yin-Yan et al., 2009).

3. **Epigenetic pathway;** emerging evidence has advocated DNA methylation status involvement in oestrogen signalling. Defective chromatin architecture at the ER target locus may have a key role in endometrial proliferative disease (Koike et al., 2015). Unlike breast cancer where increased ESR1 DNA methylation has been described as age associated promoters; age independent hypermethylation of ESR1 and PRB promoters has been reported in 90% of EC (Sasaki, Dharia, et al., 2001; Sasaki, Kotcherguina, et al., 2001; Campan et al., 2006). Furthermore, hypomethylation with subsequent activation of oestrogen downstream PAX2 has been described in EC and associated with increased tumour growth *in vivo* (Wu et al., 2005).
4. **Mutagenetic pathway;** oestrogen associated genotoxicity and endometrial tumour initiation have been proposed as a consequence of metabolic activation to catechol-oestrogens and semiquinolones and quinolones (Liehr, 2000). Several lines of evidence have shown that 4-hydroxylated oestrogen, catalysed by cytochrome P450 1B1 is able to induce DNA damage (Hayes et al., 1996). By contrast, the major hepatic and extrahepatic metabolism of E2 catalysed by P450 3A and 1A enzymes result mainly in nontoxic 2-hydroxylated oestrogen (Aoyama et al., 1990; Kerlan et al., 1992). Interestingly, recent studies have observed an increase in carcinogenic catechol oestrogens with prolonged unopposed exposure to oestrogen which was associated with DNA damage at a specific DNA region (codon 130/131) on PTEN, where mutations have frequently been found in EC (Benecke et al., 2000; Teng et al., 2013; Ke et al., 2015).

1.3.6.2 Progesterone

Progesterone is a steroid hormone essential for coordinating normal female reproductive physiology (Graham & Clarke 1997). In non-pregnant women, it is secreted by the ovarian corpus luteum that develops after ovulation. Progesterone has diverse effects on reproductive tissues, as well as in different cell types within the same tissue. The protective role against EC is well established, yet progesterone promotes development and growth of breast cancer and uterine

fibroids (Poole et al., 2006; Chlebowski et al., 2009; Ishikawa et al., 2010). This unique regulatory effect might be explained by the topographic distribution of progesterone cognate receptor in the endometrium where both epithelial and stromal compartments express PR together with associated endocrine/paracrine cross-talk (Kim et al., 2013). The unopposed oestrogen effect observed in EC and preceding hyperplasia is associated with abnormal overgrowth of endometrial glands in relation to the stroma. The role of stroma in the antiproliferative action of progesterone on the endometrial epithelium has been illustrated in a tissue recombination study utilizing PR knockout (PRKO) mice, shown to be insensitive to the inhibitory action of progesterone or oestrogen-induced endometrial hyperplasia (Kurita et al., 1998). In another mouse model $gr^{flox/flox}/Wnt7A-Cre^+$, with selective inactivation of endometrial epithelial PR, the progesterone failed to inhibit oestrogen-induced epithelial proliferation. Interestingly, the epithelial PR-null endometrium showed defective stromal decidualisation owing to the loss of Indian hedgehog expression in epithelial cells (Franco et al., 2012).

Thus, three possible mechanisms were proposed to explain progesterone-oestrogen antagonism:

1. Progesterone downregulates ER α expression and subsequently affects gene transcription (Lane et al., 1988).
2. Progesterone blocks the production of growth factors by stromal cells via a transcriptional factor called Hand2. Hand2 represses fibroblast growth factors which act as paracrine mediators to induce proliferation of the epithelium in response to oestrogen (Li et al., 2011).
3. Progesterone inhibits apoptosis of endometrial epithelial cells through PR action in stromal cells (Kurita et al., 2001).

Taken together, it is evident that there is a coordinated and intimate interplay between epithelial and stromal cells that is essential for the endometrium to proliferate, remodel, and shed in response to oestrogen and progesterone.

1.3.6.3 Androgen

Androgens are hormones produced by both the adrenal glands and the ovarian thecal cells in women. Fifty percent of testosterone is produced by the ovaries and adrenal glands and released directly into the blood stream. The other fifty percent is made from the conversion of adrenal androgens to testosterone in other parts of the body (Richard, 2007). The adrenal glands produce pre-androgens; dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) which are the main source of both testosterone and oestrogen production after menopause (Liu et al., 1990).

Historically androgens were used in ameliorating various gynaecological disorders; this has been an area of debate over the last eight decades. Despite virilising side effects, synthetic testosterone administration in dysfunctional uterine bleeding, dysmenorrhea, and menopausal sexual dysfunction has not been completely discontinued (Huffman, 1940; Kingsberg et al., 2008; Traish et al., 2009). In the early 1990s, DHEA was introduced as a therapeutic option for PM symptoms (Morales et al., 1994; Elraiyah et al., 2014). Importantly, androgen administration in healthy women does not seem to be associated with a hyperplastic or malignant transformation of the endometrium (Davis and Braunstein, 2012). Recent reports have shown a persistent atrophic phenotype in PM endometria after 52 weeks of local or oral DHEA treatment (Portman et al., 2015). Other studies have reported similar atrophic effects in the endometrium of women on transdermal testosterone (O'Donnell et al., 2012). Endometrial tissue from transgender women on high dose testosterone for at least three years prior to a hysterectomy showed atrophic glands with low ki67 expression (Perrone et al., 2009).

Several studies have shown an increased circulating androgen level in EC patients compared to normal PM, nonetheless, the association of circulating androgen levels with EC risk was not maintained after adjusting for their

circulating or local oestrogen levels (Allen et al., 2008). There are two pathways proposed to regulate the influence of androgens on endometrial tissue:

1. Androgenic pathway: different androgens bind to androgen receptors with different efficiency, and control the transcription of specific genes.
2. Oestrogenic pathway: androgens are aromatised to oestrogens or metabolised to compounds which readily bind ERs and induce transcription of mitogenic genes.

Unlike normal endometrium, in EC, the dominance of the second pathway has been postulated, based on the following reasons: the abundance of local aromatase in the endometrium (Bulun et al., 2005; Gao et al., 2014); high aldo-ketoreductase (Zakharov et al., 2010) enzymes in the neoplastic endometrial cells; and higher affinity of oestrogens to their cognate receptors compared with androgens to AR. Emerging reports have shown AR to be a positive prognostic indicator and loss of AR to be associated with a shorter disease free survival (Tanaka et al., 2015), hence an active/ intact androgenic pathway as a therapeutic target for EC requires further investigation.

1.3.7 FIGO staging

Tumour stage represents the extent of the spread of the disease at the time of diagnosis which is an essential prerequisite to planning treatment and determining prognosis. In a study of 621 patients, Gynaecologic Oncology Group (GOG33) found that 9% of patients who had clinically determined stage I disease (assessed by physical examination and imaging) had pelvic nodal metastases, 6% had para-aortic lymphadenopathy, 5% had spread to adnexa, and 6% had other extrauterine metastases at the time of surgery; therefore, surgical staging has been proposed as a gold standard for EC (Creasman et al., 1987). Complete/comprehensive surgical staging requires hysterectomy and bilateral salpingo-oophorectomy, as well as assessment of the pelvic para-aortic lymph nodes (Rungruang and Olawaiye, 2012). The debate regarding the necessity for

a complete/comprehensive surgical staging of all women with endometrial adenocarcinoma has not been resolved with some favouring restriction of lymphadenectomy to those at intermediate or high risk of nodal spread (Chan et al., 2007; ASTEC study group et al., 2009). The International Federation of Gynaecology and Obstetrics (FIGO) revised EC staging in 2009 (Table 1.5), incorporating important prognostic variables regarding parametrial involvement and lymph node status to improve its prognostic evaluation (Ellenson et al., 2011; Buhtoiarova et al., 2016). Nonetheless, 2009 FIGO staging has been criticised for eliminating the most favourable group with no myometrial invasion and did not improve its predictive ability over the 1988 system for stage I EC which constitute the vast majority of the cases (Abu-Rustum et al., 2011). Recently, incorporating histological subtype (modified dualistic), grade, myometrial invasion, and whether lymph nodes were removed has been proposed to be added to the stage I EC, aiming to improve the predictive ability over the 2009 FIGO staging (Barlin et al., 2013).

Table 1.5 FIGO staging system 2009 (Ellenson et al., 2011).

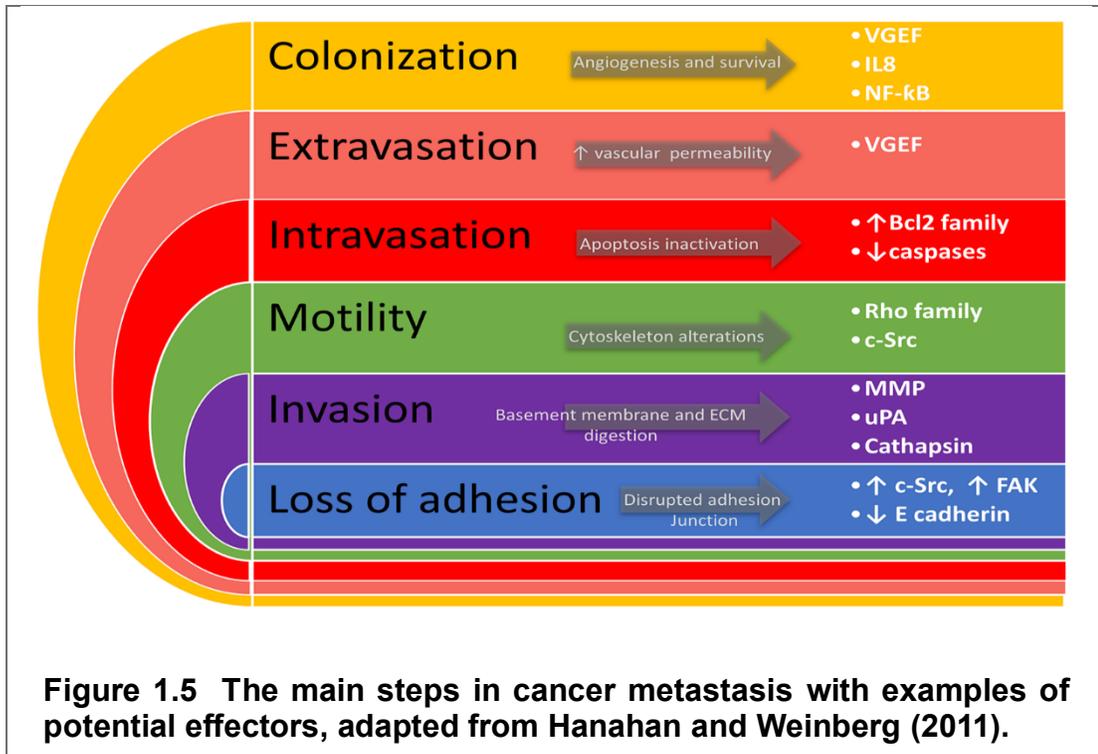
Stage	Description	
I Tumour confined to the corpus uteri	a No or less than half myometrial invasion	
	b Invasion equal to or more than half of the myometrium	
II	Tumour invades cervical stroma, but does not extend beyond the uterus	
III Local and/or regional spread of the tumour	a Tumour invades the serosa of the corpus uteri and/or adnexae	
	b Vaginal and/or parametrial involvement	
	c	1 Positive pelvic nodes
		2 Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
IV Tumor invades	a Tumour invasion of bladder and/or bowel mucosa	
	b Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes	

1.3.8 Endometrial cancer progression

Malignant cells are the result of accumulating genetic alterations. At the early stage of primary tumour expansion; malignant cells are not invasive or metastatic. Invasiveness and metastatic ability appear as a result of further accumulation of genetic alterations in the early cancerous cells. Less than 2% of primary tumour cells are considered to be highly metastatic (Luzzi et al., 1998), thus, molecular changes in these “specialised” cells with metastatic ability are expected to be higher than non-metastatic cells (Yokota, 2000).

The process of metastasis is extremely complex and dependent on the interactions between cancer cells and the ectopic microenvironment (Hanahan and Weinberg, 2011); requiring the orchestration of multiple genetic and epigenetic changes in the evolving cancer cell (Chambers et al., 2002; Fidler, 2003); Figure 1.5. Tumour cells initially lose attachment with their local surrounding cells and invade beyond the locality via degrading the extracellular matrix. With sustained survival and acquired motility, malignant cells can then enter the lymphatics and/or blood vessels in a process called intravasation. Subsequently, tumour cells sequester from the circulation (extravasate), attach and enter new tissue where they settle, proliferate and develop a supporting niche for further growth. This biological cascade of metastasis is fuelled by a group of genes involved in tumour progression (Kang et al., 1998; Su et al., 1999).

An in depth understanding of the clinical, pathological and epidemiological patterns of EC metastasis and recurrence are essential for planning therapeutic strategy. Equally, understanding molecular changes resulting in the metastatic potential is pivotal not only for identifying potentially new therapeutic targets, but also for understanding the molecular mechanism of therapy failure, improving disease monitoring and searching for new molecular markers to predict survival after relapse. This will facilitate moving towards tailored treatments for individual patients based on their genetic signatures and clinical characteristics.



1.3.8.1 Endometrial cancer metastasis

A hallmark of cancerous cells is their ability to invade the local tissue and to migrate from their initial site of origin and colonise at distant, new locations in the body. According to the Surveillance, Epidemiology and End Results (SEER) data collected from 2003–2009, 68% of EC cases were diagnosed at an early stage, but a considerable proportion (28%) were diagnosed after regional or distant spread (Siegel et al., 2012). Since EC is associated with abnormal (usually PM) bleeding, an early and obvious symptom, prompting women to seek medical help, most advanced cases are unlikely to be attributable to delay in diagnosis. Therefore, a subset of different histological subtypes with a more aggressive behaviour and a higher propensity to metastasis would be a more plausible explanation for at least some of the advanced EC (Binder and Mutch, 2014). Most ECs metastasise by invading myometrial lymphovascular spaces and spreading to regional lymph nodes. Occasionally, EC extends through the full thickness of

the uterine wall to the serosal surface with the potential to involve the parametrium or other peritoneal structures. However, uterine serous carcinomas metastasise frequently to peritoneal surfaces even when only minimally invasive (Ellenson et al., 2011). The lumen of the fallopian tube provides a potential conduit to transport neoplastic cells directly into the peritoneal cavity (Snyder et al., 2006; Stewart et al., 2013), therefore, transtubal spread has been proposed as another mechanism in serous endometrial tumours.

1.3.8.1.1 Sites of metastasis at the time of diagnosis

1.3.8.1.1.1 Local metastasis

Myometrial invasion Local invasion of malignant epithelial cells to the myometrium is an early tumour progression sign. The depth of myometrial invasion is one of the independent predictors of lymph node metastasis and tumour recurrence (Gilani et al., 2014). After the primary surgery, tumours invading more than 50% of the myometrium have been found in approximately half of HGEC, distributing equally between subtypes; whereas 10% of LGEC deeply invade the myometrium (Barlin et al., 2013). The depth of myometrial invasion is more valuable for predicting lymph node metastasis than tumour-free myometrial thickness. The tumour-free myometrial thickness provides a better prediction for adnexal involvement (Ozbilen et al., 2015). Emerging data has proposed the pattern of myometrial invasion as a possible predictor of unfavourable outcome. Certain patterns of invasion such as microcystic elongated and fragmented gland (MELF) and single cell/cell cluster invasion (SCI) patterns have been associated with shorter median survival of patients with LGEC (Euscher et al., 2013). Further studies, however, are required to confirm these data.

Lymphovascular space invasion (LVSI): Venous or lymphatic invasion is defined by the presence of neoplastic cells within endothelial-lined channels (Ellenson et al., 2011). Vascular invasion is a relatively uncommon finding in EC (about 10–20% of cases) (Nofech-Mozes et al., 2008), but the frequency

increases with deeper myometrial invasion, aggressive cell types, and decreasing histologic differentiation (Seidman and Chauhan, 2003); although an earlier study has reported a significant correlation between vascular invasion and tumour recurrence which was independent of tumour differentiation and the depth of myometrial invasion (Gal et al., 1991).

Cervical glandular and stromal invasion: EC presenting with cervical invasion (stage II) accounts for 7–15% of all EC cases each year (Ferriss et al., 2010). Several retrospective studies confirmed that cervical stromal invasion was a risk factor for overall survival and associated with an elevated risk of recurrence and an overall relapse rate of 16% (Ellenson et al., 2011). In contrast, the invasion of cervical glands has not shown an increased risk over stage I, therefore it is considered, according to FIGO 2009 as stage I (Creasman, 2009). The higher recurrence rate with cervical stroma involvement is not surprising since it is often associated with increasing grade, depth of invasion, and tumour volume, hence, after adjustment for other risk factors, the prognostic significance of cervical invasion by endometrial adenocarcinoma is limited and the evidence conflicting (Ellenson et al., 2011).

1.3.8.1.1.2 Ovarian metastasis

Ovarian metastasis of EC is diagnosed when certain criteria are fulfilled (Ulbricht and Roth, 1985);

- One Major criterion - a multinodular ovarian pattern is present
- Or two minor criteria - small (<5 cm) ovary(ies), bilateral ovarian involvement, deep myometrial invasion, vascular invasion, and tubal lumen involvement

The incidence of ovarian metastasis in women with clinical stage I EC has been reported by most studies to be approximately 5% (Creasman et al., 1987; Takeshima et al., 1998; Gemer et al., 2004). This typically presents as enlarged masses or gross adnexal abnormalities that can be detected clinically or at the

time of surgery with less than 1% adnexal involvement present as microscopic metastases (Creasman et al., 1987; Lin et al., 2015). Up to 90% of ovarian metastases of EC are reported to be of the endometrioid type, 60% of which are well differentiated with a relatively low (15%) probability of recurrence. For those patients with microscopic ovarian metastasis, EC has been indicated to be of G3 with deep myometrial invasion, and extrauterine involvement of either cervix or lymph nodes (Zaino et al., 2001). The two postulated pathological pathways of ovarian metastasis are trans-tubal implantation and lymphatic spread. Positive peritoneal cytology in the absence of nodal metastasis and concurrent metastasis to the fallopian tube are supporting the trans-tubal route. With the lymphatic spread, lymphatic nodes are often concomitantly involved while peritoneal cytology is rarely positive (Takeshima et al., 1998).

Synchronous endometrial and ovarian cancers have been reported to occur in 9–25% of premenopausal cases (Walsh et al., 2005; AlHilli et al., 2014). These patients have a favourable outcome compared to primary ovarian cancer or advanced EC (Selvaggi, 2000; Colombo et al., 2013). Endometrioid is again the most common histotype (Zaino et al., 2001; Juhasz-Böss et al., 2012). It is not clear yet whether these synchronously detected tumours represented multiple primary tumours or carcinomas arising in endometrium or ovary that metastasised to the other site. Zaino et al. (2001) observed the presence of EH and the absence of myometrial invasion which are features more commonly seen in carcinomas arising in that site, whereas vascular invasion was rare. On the ovarian side, endometriosis was present in the ovary in 31% of cases. These observations suggest that many of the cases represent the simultaneous development of carcinoma in both the endometrium and the ovary.

1.3.8.1.1.3 Fallopian tube metastasis

EC cells invading fallopian tube walls have not been described individually, but they have as part of the uterine adnexa metastasis, with a recorded rate of 5% in all EC cases (Creasman et al., 1987). Interestingly, many studies have observed

EC cells in the tubal lumen and proposed the fallopian tube as a potential method of EC spread to the peritoneal cavity, particularly in serous EC (Dahle, 1956; Creasman and Lukeman, 1972). Furthermore, these intra-luminal EC deposits have been associated with HGEC and have the capacity to implant and invade the peritoneal cavity in the absence of deep myometrial invasion (Snyder et al., 2006; Stewart et al., 2013).

1.3.8.1.1.4 Parametrial metastasis

Parametrium is the connective tissue that surrounds the supravaginal portion of the cervix. The description of parametrial invasion (stage IIIB) of EC is limited to five studies in the literature. These studies have concluded that parametrial invasion was correlated significantly with deep myometrial invasion, retroperitoneal lymph node metastasis, ovarian metastasis, cervical involvement, positive peritoneal cytology results, and LVSI (Yura et al., 1996; Sato et al., 2003; Watanabe et al., 2010). Moreover, Tamussino et al. (2000) and Lee et al. (2010) have shown in retrospective studies that cervical stromal invasion was a predictor of parametrial invasion in clinical stage II patients who underwent a radical hysterectomy. Interestingly, an association between parametrial invasion and LVSI has been observed in stage IA EC when deep myometrial invasion was absent, suggesting the invasion was through lymphatics rather than direct (Watanabe et al., 2010). Parametrial spread was associated with unfavourable patient outcomes (Yura et al., 1996; Watanabe et al., 2010); however it was not confirmed as an independent prognostic parameter (Watanabe et al., 2010).

1.3.8.1.1.5 Vaginal metastasis

Vaginal metastasis is staged as FIGO IIIB, however, specific records describing the extension of EC to the vagina are lacking in the literature.

1.3.8.1.1.6 Lymph node metastasis

Regional lymph nodes metastases (FIGO stage IIIC) account for 5-20% of all EC and are associated with an approximately 50% probability of recurrence and poor survival (Mundt et al., 2001). Information from the SEER registry suggested that the incidence of pelvic and para-aortic lymph node metastases is less than 1.8% in clinical stage I endometrioid adenocarcinoma whereas patients with FIGO stage IC–IIB, grade 2–3 EC have a higher risk of lymph node involvement (Katsoulakis et al., 2014). Occult malignant cells invading pelvic LN are postulated to contribute in early stage EC recurrence, yet further studies are required to confirm that (Todo et al., 2016). About 10% of women with endometrioid endometrial adenocarcinoma have metastasis to pelvic lymph nodes (Chi et al., 2008), nearly a third of them will also have positive para-aortic lymph nodes (LN). Among the extrauterine risk factors, the presence of positive para-aortic LN is the most important factor in predicting prognosis (Ellenson et al., 2011). A recent retrospective study included approximately 800 EC patients has indicated that para-aortic LN metastasis was higher in nonendometrioid compared with endometrioid cancer (Haltia et al., 2014) contradicting a previous prospective study of 422 participants which failed to show difference in the pattern of retroperitoneal spread with different histological types (Mariani et al., 2008). Cohort characteristics, number of participants, and primary management plan are likely to be the reason for this discrepancy. Recurrence within 5 years has been reported in 64% of patients with positive para-aortic LN compared with 15% with negative para-aortic LN (Ellenson et al., 2011).

1.3.8.1.1.7 Distant metastasis

Distant metastasis at diagnosis represents no more than 7% of all EC (Cancer research UK, 2016) most frequently involving the omentum, peritoneum, extra-pelvic LN and the lungs. Nonendometrioid sub-type and multiple extraperitoneal metastatic sites have been identified as predictors of unfavourable patient survival, which unfortunately rarely reaches 12 months (Tanioka et al., 2010).

1.3.8.2 Endometrial cancer recurrence

Recurrence is defined as the local, regional or distant growth of the tumour after a period of complete remission (Ellenson et al., 2011). Recurrence can develop as a result of incomplete resection of the tumour or due to the emergence of resistant malignant cells that can survive the adjuvant therapy (Odagiri et al., 2011). High grade and advanced stage EC are at a higher risk to develop extra-pelvic secondaries within 3 years (Kurra et al., 2013). Shorter disease free period and multiple sites of recurrence are indicators of poor patient outcome. The median survival of patient with recurrent EC does not exceed 12 months (Odagiri et al., 2011). Metastatic ECs do not have tropism to specific organs. By contrast, they can establish in any tissue. Common sites of EC metastasis are:

1.3.8.2.1 Vaginal recurrence

The vagina is the most common site of EC recurrence with 2.9% of the stage I cases developing vaginal relapse within 5 years (Mariani et al., 2005; Bjelakovic et al., 2014). Vaginal relapse has been previously associated with Grade 3 tumours, lymphovascular invasion, and nonendometrioid type carcinomas (Mariani et al., 2005); however, a more recent report suggested cervical tumour invasion as an independent predictor of recurrence at this site. According to that particular study, most cases were FIGO Grade 2 at the time of hysterectomy and retained the same differentiation at the time of recurrence. Furthermore, deaths were not observed among patients with isolated vaginal recurrence, suggesting that vaginal recurrence is not a marker of aggressive tumour biology (Moschiano et al., 2014).

1.3.8.2.2 Lymph node recurrence

Lymph nodes are the most common site of recurrence overall and the second common site after vaginal vault in single site recurrence. Up to 32% of LN might develop recurrence after radiotherapy (Greven et al., 1989). Mariani et al (2002) indicated that positive LN at surgery is an independent predictor of lymphatic

recurrence, along with the presence of LVSI and cervical stromal invasion, although this might be greatly influenced by postsurgical therapeutic modality used (Sohaib et al., 2007).

1.3.8.2.3 Distant recurrence

The peritoneum and the lungs are the typical distant recurrence sites of EC; nonetheless, intra-abdominal organs, bones, and the brain are increasingly identified due to improved imaging techniques such as transvaginal ultrasonography, MRI/CT and PET/CT (Sohaib et al., 2007). Haematogenous spread occurs most often to the lungs, liver, bone and brain. Peritoneal carcinomatosis is observed in 28% of recurrent EC, making it one of the most typical sites (Blecharz et al., 2011) whereas the spleen, pancreas, rectum, and muscle are unusual recurrence site of EC, with less than 5% of reported disease (Kurra et al., 2013).

1.3.9 Endometrial cancer and biomarkers

A tumour biomarker is a molecule produced by a tumour or by the body in response to a tumour which provides help in cancer detection and/or guides management plan (Brünner et al., 2008). Biomarkers can be divided into: (1) *screening*: assist with early detection of malignant changes; (2) *prognostic*: help in assessing the risk of recurrence and subsequent death of the individual patient following intended curative surgery of the primary tumour; (3) *predictive*: aid in estimating the likelihood of obtaining an objective response to a specific form of anti-cancer therapy and finally (4) *monitoring markers*: serve as post-operative surveillance of the cancer patients as their levels in blood may increase prior to the clinical detection of tumour recurrence (Brünner et al., 2008). Out of these four categories, biomarkers implicated in EC are largely limited to prognostic markers (Engelsen et al., 2009; Binder and Mutch, 2014; Li et al., 2015; Minář et al., 2015; Secord et al., 2015), Figure 1.6.

Although stage, grade, depth of invasion, LVSI, and histological subtype are the main clinical and pathological prognostic factors at diagnosis, stratification of the patients in different clinical trials varies according to the used definition of risk group in that trial as shown in Table 1.6. This discrepancy causes inconsistent recommendations of postsurgical (chemo/ radio/or endocrine therapy) especially in the absence of reliable predictive and monitoring biomarkers.

In addition to the clinical and pathological prognostic markers, many studies suggested molecular markers which are differentially expressed in one type of EC than the other (Engelsen et al., 2009; Binder and Mutch, 2014; Li et al., 2015; Minář et al., 2015; Secord et al., 2015); Figure 1.6. Some of these markers have been implicated in the identification histological subtypes when

	Clinicopathological	Molecular	Others
Consistently reported	<ul style="list-style-type: none"> • Age • Histological subtype • FIGO stage • Tumour grade • LVSI 	<ul style="list-style-type: none"> • ER • PR • P53 • Ki67 • Bcl-2 	<ul style="list-style-type: none"> • DNA Ploidy
Inconsistently reported	<ul style="list-style-type: none"> • Race 	<ul style="list-style-type: none"> • HER-2/neu • PTEN • P16 • MSI • βcatenin • Kras 	
Few reports	<ul style="list-style-type: none"> • BMI? 	<ul style="list-style-type: none"> • AR • POLE • Angiogenesis markers eg VEGF • E-cadherin • P13K signal activation • S100A4 	<ul style="list-style-type: none"> • serum CRP • serum D-dimer* • Serum human epididymis protein 4 (HE4) • CA125 • L1CAM

Figure 1.6 Prognostic predictors and biomarkers for endometrial cancer, figure adapted from Binder and Mutch (2014). *A venous thromboembolism factor.

the morphological features are not distinctive enough to make the diagnosis or when the origin of the primary tumour is difficult to determine. Recently, with the appearance of targeted therapy, new predictive tests are expected to be developed and validated in clinical research studies.

As endocrine therapy is one of the therapeutic options in EC, the hormone receptor status of ECs would be expected to play a role in predicting clinical outcome and guiding therapeutic choice (Zhang et al., 2015). Unlike breast cancer (Anonymous, 1994), steroid receptor status is not routinely reported for EC and the literature on the prognostic value of ER α , ER β , ER α /ER β ratio and PR in EC is inconsistent (Takama et al., 2001; Fujimoto et al., 2002; Shabani et al., 2007; Jongen et al., 2009; Zannoni et al., 2013). In this section a brief description of types, structure, and function of the prognostic factors of interest, steroid receptor and metastasis inducing proteins, will be discussed as well as a critical review of the available evidence in EC.

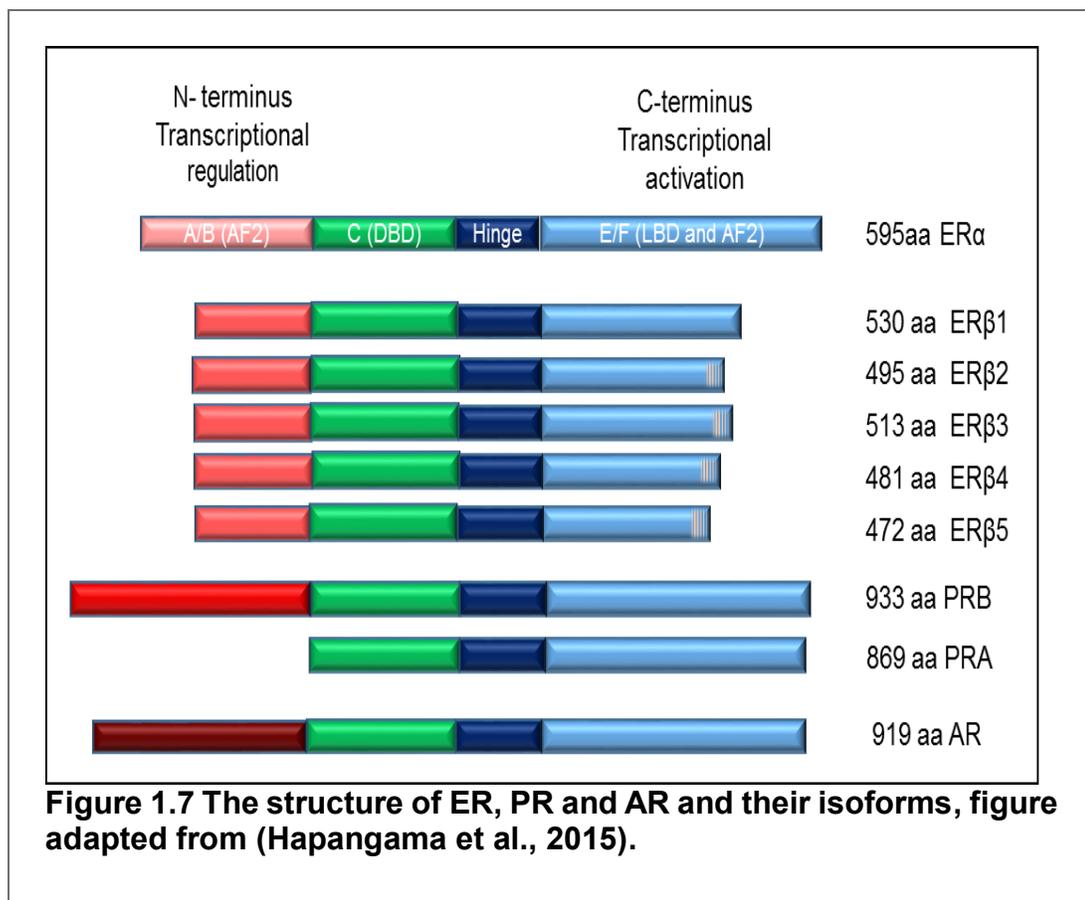
Table 1.6 The variation in risk factors classifications according to trials or society guidelines, adapted from Morice et. al. (2014)

	PROTEC	GOG99	SEPAL	ESMO	ESMO modified	Simplified\$ criteria	ESMO-ESGO-ESTRO Consensus
Low risk	<ul style="list-style-type: none"> Stage IA, G1 Type I 	<ul style="list-style-type: none"> Stage IA 	<ul style="list-style-type: none"> Stage IA or IB type I + no LVSI 	<ul style="list-style-type: none"> Stage IA, G1,2 type I 	<ul style="list-style-type: none"> Stage IA, G1,2 type I + no LVSI 	<ul style="list-style-type: none"> Stage IA 	<ul style="list-style-type: none"> Stage IA, G1,2 type I, + no LVSI
Intermediate risk	<ul style="list-style-type: none"> Stage I+ G 1,2 type I + >50% MIV >60 years + >50% MIV G3 type I + <50% MIV 	<ul style="list-style-type: none"> Stage IB, IC, II + ≤ 50year + ≤2 Pathological RF* 50-69 year + ≤1 pathological RF* ≥70 year + no pathological RF* 	<ul style="list-style-type: none"> Stage IA + G3 type I or II ± LVSI Stage IB, G 1, 2 type I ± LVSI Stage IB+G3 Type I or II ±LVSI Stage IC, stage II, any grade ± LVSI 	<ul style="list-style-type: none"> Stage IA +G3 type I Stage IB G1,2 type I 	<ul style="list-style-type: none"> Stage IA G1, 2 type I+ LVSI Stage IA G3 type I + no LVSI Stage IB G1,2 type I + no LVSI 	<ul style="list-style-type: none"> IB , II + no pathological RF 	<ul style="list-style-type: none"> Stage IA G3 type I ± LVSI
Intermediate high risk	-----	<ul style="list-style-type: none"> Stage IB, IC, II + Any age + 3 Pathological RF* 50-69 year + ≥2 pathological RF ≥70 year + ≥ 1 pathological RF* 	-----	-----	<ul style="list-style-type: none"> Stage IA G3 type I + LVSI Stage IB G1,2 type I + LVSI Stage IB G3 + no LVSI Stage IB G3 type I + LVSI 	<ul style="list-style-type: none"> IB , II + Any age + ≥ pathological RF 	<ul style="list-style-type: none"> Stage I G1,2 type I + LVSI ± <50% MIV
High risk	<ul style="list-style-type: none"> Stage III-IV Type II 	<ul style="list-style-type: none"> III IV 	<ul style="list-style-type: none"> Stage III or IV, any grade, any LVSI 	<ul style="list-style-type: none"> Stage IB +G3 type I Type II of all stages 	<ul style="list-style-type: none"> Type II all stages 	<ul style="list-style-type: none"> III IV 	<ul style="list-style-type: none"> Stage IB G3 type I ± LVSI Stage II Stage III type I + no residual disease Type II
Advanced and metastatic	-----	-----	-----	-----	-----	-----	<ul style="list-style-type: none"> Stage III residual disease and stage IV A and B

PORTEC, Post-Operative Radiation Therapy in Endometrial Carcinoma; GOG, Gynaecologic Oncology Group adjuvant radiation for intermediate-risk endometrial cancers; SEPAL, Survival Effect of Para-Aortic Lymphadenectomy in endometrial cancer. ESMO, European Society for Medical Oncology; ESGO, European Society of Gynaecological Oncology; ESTRO, The European Society of Radiotherapy and Oncology; §based on FIGO 2009 staging system; G, grade; MIV, myometrial invasion; LVSI, lymphovascular space invasion. RF, risk factors (*grade 2 or 3 histology, positive LVSI, myometrial invasion to outer third). (Colombo et al., 2015; Kong et al., 2015; Morice et al., 2015)

1.3.9.1 Steroid receptors

Steroid receptors are members of the nuclear hormone receptor superfamily and share the common, evolutionarily conserved structural and functionally distinct domains as the other members of the superfamily; Figure 1.7. This includes a central, highly conserved DNA binding domain (DBD), which binds to the same ligand responsive element in the target gene promoters; multifunctional ligand-binding domain (LBD) at the C-terminal; the ligand-dependent AF-2 at the C-terminal; constitutively active AF-1 at the N-terminal and flexible-hinge D-domain in between LDB and DBD.



1.3.9.1.1 ERs

The human ER gene ESR1 was first cloned in 1996 from testis (Mosselman et al., 1996). Interestingly the pivotal accepted role of ERs in reproduction is an evolutionarily late development as the evidence suggests that in early order invertebrates the reproductive role of E2 is not mediated by the ER and may take place through ancient, ER-independent pathways (Keay et al., 2006). Despite their close homology, ER β (ESR2) gene is located on chromosome 14 whereas ER α protein is coded by a different gene (ESR1) located on chromosome 6. ER β can have opposing action on the same gene promoter, in response to E2. These inhibitory effects of ER β on ER α activity may be exerted through a combination of altered recruitment of key transcription factors and increased ER α degradation. In contrast, ER β expression is induced by E2 acting via ER α and may be suppressed by hypermethylation of ER β promoter (Rody et al., 2005). The proliferative function of ER α is essential for reproduction, yet is associated with obvious E2-associated health risks in the endometrium (and in other organs). Available data on ER α and β proteins and mRNA expression in EC subtypes are largely confined to endometrioid EC (Hapangama et al., 2015). The general consensus that the carcinogenesis process in Type II ECs is E2-independent is likely to be the reason for the lack of data on ER β expression in Type II ECs in particular, with virtually no studies examining the ER β expression in serous and clear cell carcinoma.

ER α splice variants have been described in the normal and malignant endometrium. Exon skipping variety constitutes the majority of ER α splice variants, out of which ER $\alpha\Delta 5$, ER $\alpha\Delta 4$ and ER $\alpha\Delta 7$ are the most studied in the endometrium. Overall, more ER α splice variants are found in malignant tissues compared with normal or premalignant endometrial tissues (Witek et al., 2001; Taylor et al., 2010).

Similar expression patterns to ER α have been reported for ER β variants (ER $\beta 1$, ER $\beta 2$, and ER $\beta 5$) in endometrioid EC samples: ER $\beta 1$ and ER $\beta 2$ immunoexpression was higher in low-grade ECs, whereas ER $\beta 5$ expression was

constitutively intense regardless of the grade (Collins et al., 2009). In tamoxifen-associated ECs, ER β expression was particularly prominent compared with spontaneous ECs (Negoita and Mihailovici, 2011). ER β 1 and ER β 2 expression did not correlate with tumour grade, FIGO stage or myometrial invasion, but the ER α /ER β ratio was reported to be an independent prognostic factor of overall survival (Zannoni et al., 2013). The ER α /ER β ratio has been used to evaluate the imbalance in the relative expression of ER isoforms in endometrial (Fujimoto et al., 2000; Jazaeri et al., 2001; Takama et al., 2001; Jongen et al., 2009; Smuc and Rizner, 2009) and other hormonal-regulated malignancies, such as breast cancer, and has been suggested as a potential predictor of patient outcome (Zannoni et al., 2013).

Interestingly, ER has been reported as the best predictor of response to sequential endocrine therapy (MPA/ tamoxifen) for patients with advanced or recurrent EC whereas PR showed limited value (Singh et al., 2007) probably because ER is required to maintain PR level for binding with MPA.

1.3.9.1.2 PRs

PR was first purified and cloned in 1975 (Smith et al., 1975). Two protein isoforms have been identified, PR-A and PR-B, that are produced from a single gene by transcription from two distinct promoters (Conneely et al., 1989, 2001; Kastner et al., 1990). These two isoforms are conserved in a number of vertebrate species including humans and rodents (Lessey et al., 1983; Conneely et al., 1989; Shyamala et al., 1990; Bethea and Widmann, 1998). The human PR-B is the larger isoform by an additional 164 amino acids at the amino terminus (Lessey et al., 1983; Gronemeyer et al., 1991) as shown in Figure 1.7. In endometrium, the ratios of the individual isoforms vary according to the reproductive, hormonal status (Duffy, 1997; Mangal et al., 1997) and during carcinogenesis (Graham et al., 1996).

PRB acts as an activator of progesterone-responsive genes, whereas PRA is transcriptionally inactive. In addition, PRA also functions as a strong trans-

dominant repressor of PRB as well as the human ER transcriptional activity (Vegeto et al., 1993). The precise mechanism underlying the differential activities of the two PR isoforms is not fully understood. Studies have suggested that the conformations which PRA and PRB adopt within the cell may contribute to its different transactivation functions. Further, studies have described that activated PRA is unable to efficiently recruit the coactivators SRC-1 and GRIP1 (Giangrande et al., 2000). by contrast, it interacts efficiently with the corepressor SMRT permitting it to function as a trans-dominant repressor (Giangrande et al., 2000).

In vitro, it has been shown that PRA is localised to the nucleus of EC cells even in the absence of progesterone while PRB is more abundant in the cytoplasm and requires the ligand for nuclear translocation (Leslie et al., 2005). Generally, PR expression is down-regulated in less differentiated ECs; nonetheless, the debate about isoforms expression is ongoing with one study suggesting that both isoforms are lost in advanced EC a second pointing out that the relative expression of the isoforms alter in advance stage (Arnett-Mansfield et al., 2001) and a third indicating that PRB level is slightly higher in advanced endometrial tumours (Fujimoto et al., 1995)

The prognostic value of PR has been recognised since 1983 (Martin 1983), Although compelling evidence from pooled retrospective studies exists over the last two decades, suggesting PR to have a significant independent prognostic role (Zhang et al., 2015), the predictive value for PR guiding endocrine therapy has not yet been determined in terms of standard quantification methods and best cut-off points. Even for fertility sparing, advanced and recurrent EC, where progesterone is the first line of treatment, recent ESGO-ESMO recommendation limits PR assessment to advanced/recurrent diseases (Colombo et al., 2015). The argument against the assessment of PR in fertility sparing cases is that 2/4 (50%) patients who showed a response to progesterone were PR negative, however, 5/5 (100%) patients who responded were PR positive and the difference between the two groups was significant, $P=0.008$ (Yamazawa et al., 2007). The small

sample size (n=9) for the referenced trial and absence of confirming studies reduces the significance level of the available evidence. Also, the absence of standardised guidelines for more accurate IHC scoring and an optimised level of positivity that predicts response to treatment may increase the possibility of pooling data from different trials to generate evidence that is clinically relevant.

1.3.9.1.3 AR

AR gained interest in gynaecology early in the 1980s after the introduction of the androgenic progesterone drug danazol for the treatment of endometriosis (Traish et al., 2009). The first description of AR expression in the human endometrium was coined by Horie et al. 1992. The AR gene is located on the X chromosome at the locus Xq11-Xq12 which encodes a 110-kDa protein consisting of 919 amino acids (Lubahn et al., 1988) as shown in Figure 1.7. Multiple splice variances of AR have been described in prostate cell lines (reviewed in Sprenger & Plymate 2014), but evidence for their expression in normal and malignant endometrial tissue is yet to be investigated. Studies describing AR expression in endometrioid EC are scarce and inconsistent with virtually no reports on expression in non-endometrioid subtypes. However emerging evidence has indicated a potential prognostic role of AR (Tanaka et al., 2015).

1.3.9.2 Metastasis inducing proteins

Metastasis inducing proteins (MIPs) name is coined for S100A4, S100P, and AGR2 and some other proteins which when overexpressed were able to induce benign breast cells to metastasise to other tissues in a rat model (Lloyd et al., 1998; Liu et al., 2005; Wang et al., 2006). Increasing evidence advocates the prognostic role of these proteins in cancer. Breast, prostate and ovarian cancer expressing higher levels of MIPs have been shown to associate with shorter overall survival times, a higher propensity for recurrence and they are more resistant to therapy. An overview of MIPs structure, biological and prognostic roles are described in this section

1.3.9.2.1 S100A4

S100A4, (previously known as: mts1, p9Ka, FSP1, CAPL, calvasculin, pEL98, metastasin) is a member of the S100 family which represents a large EF-hand Ca^{+2} binding subfamily that contains up to 22 proteins (Ravasi et al., 2004). Structurally, the S100 family are low molecular weight (10 to 12 kd) acid proteins that are characterised by two Ca^{2+} binding sites each of which is flanked by helices, the two units are linked by a hinge region as shown in Figure 1.8 A and B (Kretsinger et al., 1991). The EF motifs of S100 proteins bind Ca^{+2} at different affinity. C-terminal site affinity is found to be higher than that of the amino-terminal site (Donato, 1986).

S100A4 was first isolated and cloned in the late 1980s from a rat mammary cell line (Barraclough et al., 1987) . In human, the S100A4 protein is encoded by a gene cluster on chromosome 1q21.3 that encodes six other S100 family members. This encoding gene cluster is involved in frequent rearrangements throughout tumour progression suggesting that S100A4 expression at later stages of malignancy might be influenced by chromosomal abnormalities (Engelkamp, 1993).

Abnormal expression of S100A4 proteins has been described in non-malignant inflammatory and degenerative human diseases as well as cardiomyopathy (Schneider et al., 2008); however, its metastasis inducing property has received an increasing consideration. It has been shown that S100A4 is involved in many functions that are essential for metastasis including,

- **Cell detachment;** S100A4 involvement in cell polarity and adhesion dysregulation is thought to be through the cadherin family. Studies have shown a negative association between S100A4 and E-cadherin expression which was further confirmed when S100A4 gene was knocked down (Keirsebilck et al., 1998; Boye et al., 2010).

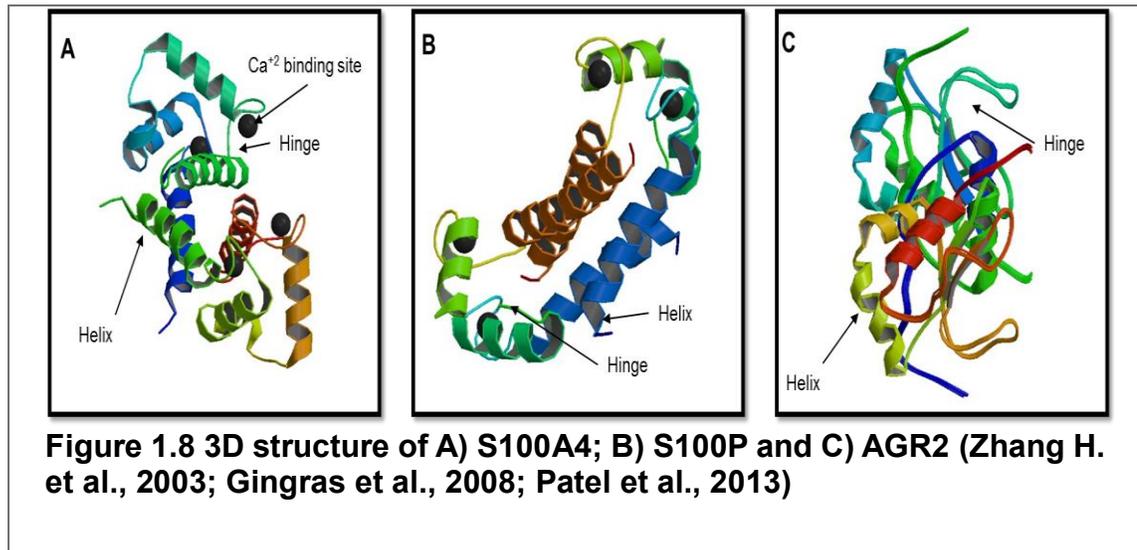
- **Invasion;** the exact mechanisms explain S100A4 induced invasion are less understood; one suggested mechanism is through degrading the extracellular matrix component. Down-regulation of S100A4 reduces the ability of highly metastatic osteosarcoma cell lines to invade Matrigel-coated filters. This was associated with a reduction of the mRNA levels of matrix metalloproteinases MMP2, and MMP1 (Bjørnland et al., 1999).
- **Cell migration;** motility and adhesion happen as a consequence of altered microfilament remodelling of the cells. The C-terminal region of S100A4 was found to have the ability to bind the heavy chain of non-muscle myosin IIA (MHC IIA) (Kriajevska et al., 1998; Ismail et al., 2008) and non-muscle tropomyosin (Takenaga et al., 1994) forming a complex with sulphiredoxin (Bowers et al., 2012), a ubiquitous antioxidant which consequently results in microfilament remodelling. In the leading edge of migration, S100A4 has been shown to co-localise with MHC IIA (Kim and Helfman, 2003; Li and Bresnick, 2006) and promote disassembly of preformed filaments (Santamaria-Kisiel et al., 2006).
- **Cell death;** direct interaction and/or reciprocal influence between p53 and S100A4 has been shown by several groups using *in vitro* binding assays. A series of *in vitro* experiments revealed that S100A4 binds to the C-terminal end of p53 and modulates transcription of p53-responsive genes, such as mdm2, Bax, p21 and thrombospondin (Grigorian et al., 1999; Boye and Gunhild, 2010). The nuclear expression of S100A4 has been found to inversely correlate with p53. Moreover, knockdown of S100A4 leads to p53-dependent cell cycle arrest and increased cisplatin-induced apoptosis (Orre et al., 2013). Another study has shown that, via interaction with p53, S100A4 differentially modulates the transcription of p53-regulated genes, such as *p21/WAF* and *bax* (Klingelhöfer et al., 2007). The frequency of apoptosis in the spleen of S100A4^{-/-} animals after whole-body gamma-

irradiation was reduced compared to the wild-type animals as well as the transcriptional activation of the p53 target genes. Thus, spontaneous tumour formation observed in S100A4 knockout model was expected to be a consequence of functional destabilisation of p53 tumour suppressor gene (Naaman et al. 2004)

- **Angiogenesis;** the expression of S100A4 by endothelial cells proposes a functional role of this protein in cancer angiogenesis which is a requisite for a metastatic embolus to proliferate and grow in the secondary tissue. S100A4 in corneas of transgenic mice was able to induce neovascularisation, with the incidence of liver haemangiomas significantly increased in these mice when they become older (Ambartsumian et al., 2001). Through annexin II, an endothelial plasminogen co-receptor, extracellular S100A4 has been shown to accelerate tissue plasminogen activator-mediated conversion of plasminogen to plasmin (Semov et al., 2005). Schmidt and colleagues were able to induce capillary-like growths in three-dimensional Matrigel cultures *in vitro* by stimulating MMP-13 production from endothelial and promoting this via extracellular S100A4 (Schmidt-Hansen et al., 2004).
- **Epithelial–mesenchymal transition EMT;** it has been hypothesised that some of the pro-metastatic actions of S100A4 could be explained by their role in epithelial-mesenchymal transition (Boye and Gunhild, 2010). Xue et al have elegantly shown that S100A4 is functionally involved in the development of lung metastasis in PyV-mT mice (Xue et al., 2003)

Stromal expression of S100A4 is increased in endometriotic lesions, with concomitant significant increased expression in corresponding late secretory phase eutopic endometrium compared to fertile controls (Hapangama et al., 2012). In EC, emerging studies have reported upregulation of S100A4 protein and transcript in HGEC compared to LGEC (Xie et al., 2007; Chong et al., 2014) which was associated with unfavourable patient outcome (Chong et al., 2014).

Furthermore, S100A4 showed no significant correlation with the hormonal status of the tumours, instead epigenetic regulation of this protein has been suggested whereby HGEC with elevated S100A4 mRNA showed no methylation of the gene whereas normal endometrial samples did (Xie et al., 2007).



1.3.9.2.2 S100P

S100P is a relatively small (95 amino acid) isomer of the S100 family, (Figure 1.8B). It was first purified from human placenta with a structure similar to that of other S100 family but unlike other family members, S100P is encoded by a gene located on human chromosome 4q16 (Becker et al., 1992).

Overexpression of S100P has been described in several malignant conditions such as breast ,prostate, pancreatic, lung, colorectal, and most recently liver cancer (Guerreiro Da Silva et al., 2000; Mousses et al., 2001; Logsdon et al., 2003; Diederichs et al., 2004; Fuentes et al., 2007; Whiteman et al., 2007) and was correlated with poor patient outcome (Wang et al., 2006). S100P has been observed in early stage pancreatic intra-epithelial neoplasms (Downen et al., 2005) and high risk breast lesions (Guerreiro Da Silva et al., 2000) suggesting that S100P has a role in tumour initiation and could potentially be a premalignant marker.

The mechanisms by which S100P exerts its metastasis inducing properties are not well known. It has been suggested that both structural conformation and binding to different types of proteins determine the subcellular distribution of S100P and subsequently reflect its functional status. Ezrin, S100PBPR, and RAGE are the binding partners that have been reported so far in S100P subcellular translocation whereby binding to ezrin promotes peripheral localisation (Koltzsch et al., 2003), binding to S100PBPR promotes nuclear localisation (Downen et al., 2005) and extra-cellular S100P binding to RAGE activate a signal transduction pathway (Mandinova et al., 1998; Hsieh et al., 2003). More recently, S100P has been identified to interact with acyBP/SIP, a 30 kDa protein and reduce the degradation of β catenin (Matsuzawa and Reed, 2001; Filipek, 2002, 2006), moreover, upregulation of S100P in a pancreatic cell line has been shown to induce precursors of cathepsin D which take part in the proteolytic degradation of the extracellular matrix, hence increase the invasive potential of the tumour (Briozzo et al., 1988; Whiteman et al., 2007).

1.3.9.2.3 AGR2

AGR2 is the human analogue of *Xenopus laevis* Anterior gradient (Shih et al., 2007) a protein that belongs to the protein disulphide isomerase family (PDI) of endoplasmic reticulum-resident proteins (Persson et al., 2005); its structure is shown in Figure 1.8C. The gene encoding AGR2 located on chromosome 7p21.3 along with those encoding AGR3 which shares 71% sequence identity (Petek et al., 2000; Fletcher et al., 2003). AGR2 mRNA is strongly expressed in endoderm-derived organs that contain mucus-secreting cells. It protects from diseases in physiological levels through regulating goblet cells transcription factors FOX1 and FOX2 (Zheng et al., 2006). Mice lacking AGR2 survived, but were highly susceptible to colitis (Park et al., 2009), whereas overexpression of AGR2 protein has been described in a variety of human cancers and detected in plasma and extracellular fluids, thus proposing a crucial role in carcinogenesis (Chevet et al., 2013). Although the exact function of PDI in cancer remains unclear, the

regulation of protein folding, maturation and secretion have been suggested to drive metastatic cell growth (Chevet et al., 2001; Moenner et al., 2007).

In mammary tissue, AGR2 is expressed in an oestrogen dependent manner during evolution (Kuang et al., 1998; Thompson and Weigel, 1998; Salmans et al., 2013). Several lines of investigations indicate that overexpression of AGR2 promotes breast tumorigenesis through increased cell survival and proliferation and inducing lung metastasis in an *in vivo* metastasis assay (Liu et al., 2005). AGR2 expression is associated with ER-positive tumours; its overexpression is a predictor of unfavourable outcomes especially in tumours that resist hormone therapy (Innes et al., 2006; Hrstka et al., 2013), which has been attributed to paradoxical induction of AGR2 by tamoxifen, an oestrogen antagonist (Hengel et al., 2011). A pro-oncogenic feature of AGR2 is demonstrated by its ability to induce proliferation, invasion and survival; the cellular mechanisms behind these functions are proposed to be through:

- EGRF /Hippo pathways: AGR2 is shown to specifically induce AREG, an EGRF ligand, that stimulate cell proliferation either through phosphorylation of EGFR and its downstream AKT (Dong et al., 2011) or through Hippo pathway repression by dephosphorylating YAP and subsequent inhibition of apoptosis and promotion of cell division (Dong et al., 2011).
- p53 pathway: Overexpression of AGR2 is shown to attenuate mutant p53 phosphorylation (Pohler et al., 2004) and regulate its intracellular distribution (Fourtouna, 2009) which suggest that AGR2 ability to promote cell survival may contribute to its activity as a p53 kinase inhibitor

Emerging data has reported that AGR2 is upregulated in the secretory phase of the eutopic endometrium of patients suffering from endometriosis compared to fertile control, which proposes that AGR2 enhances endometrial invasiveness and

may contribute to the establishment of ectopic endometriotic deposits after retrograde menstruation (Hapangama et al., 2012).

1.3.10 Endometrial cancer management

1.3.10.1 Surgical treatment

Surgery is the standard first line management for EC in both younger and older patients when the tumour is confined to the pelvis. Governed by the clinical staging and the existence of comorbidity in individual patients, the available surgery treatment options may vary from minimally invasive, to open and from limited to comprehensive. Cytoreductive surgery is being increasingly recommended as one of the multimodality approaches for advanced and recurrent disease (Colombo et al., 2015). Table 1.7 summarised the management guidelines according to tumour extent.

1.3.10.2 Adjuvant therapy

Therapeutic modalities used to maximise primary surgical effectiveness are termed as adjuvant therapy. Elderly patients with EC are usually the target for such therapy because they typically present with advanced disease and aggressive histopathology; nonetheless, only half of them actually receive it owing to poor compliance and comorbidities (Clark et al., 2016). Adjuvant therapy in EC includes:

Table 1.7 Surgical treatment of endometrial cancer (Colombo et al., 2015).

Stage I LR	➤ Hysterectomy with bilateral salpingo-oophorectomy
Stage I IR	➤ Hysterectomy with bilateral salpingo-oophorectomy ± bilateral pelvic-para-aortic lymphadenectomy
Stage I HR	➤ Hysterectomy with bilateral salpingo-oophorectomy + bilateral pelvic-para-aortic lymphadenectomy
Stage II	➤ Modified radical hysterectomy with bilateral salpingo-oophorectomy and bilateral pelvic-para-aortic lymphadenectomy
Stage III-IV	<ul style="list-style-type: none"> ➤ Complete macroscopic surgical cytoreduction and comprehensive staging. ➤ Systemic therapeutical approach with palliative surgery

Abbreviations: Low risk tumour (LR), grade 1 or 2 endometrioid cancer with myometrial invasion <50%, Intermediate risk tumour (IR), grade 1 or 2 with deep myometrial invasion ≥ 50% or grade 3 with myometrial invasion <50%, High risk (HR), grade 3 with myoinvasion ≥ 50% or nonendometrioid of any stage

1.3.10.2.1 Radiotherapy

A randomised clinical trial (PORTEC-2) comparing vaginal brachytherapy and external beam radiation in intermediate risk patients showed that the two radiation therapies were equally effective in reducing the rate of local/regional recurrence, but the quality of life was better in the vaginal brachytherapy arm (Nout et al., 2010). However, three large randomised studies failed to demonstrate that radiation improves overall or disease-specific survival (Keys et al., 2004; Blake et al., 2009; Nout et al., 2010). Radiation therapy is also the standard treatment for vaginal recurrence (external beam plus vaginal brachytherapy) with high rates of local control, complete response and a 5-year survival of 50% (Colombo et al., 2015).

1.3.10.2.2 Chemotherapy

Platinum-based chemotherapy is recommended in stage I grade3 with adverse risk factors (patient age, LVSI and high tumour volume) and in patients with stage II–III (Colombo et al., 2015). Chemotherapy is suggested to be superior to pelvic radiotherapy in patients aged >70 years with deep myometrial invasion, those with grade 3, those with stage II or those with stage I disease and positive peritoneal cytology (Colombo et al., 2013). Preliminary *in vitro* results showed that two drug regimens (carboplatin and paclitaxel) might be as good as the three drug regimen (cisplatin, doxorubicin, and paclitaxel) in terms of activity against cancer and overall survival and had the advantage of being less toxic (Ballard et al., 2010). EC recurring after chemotherapy is largely a chemo-resistant disease. Among the currently used chemotherapeutic agents only paclitaxel has consistently shown a response rate of >20% (Colombo et al., 2013)

1.3.10.2.3 Combined chemo-radiotherapy

Combined sequential use of chemotherapy and radiotherapy has been associated with a 36% reduction in the risk of relapse or death of stage I–II, IIIC, of any histology with improved cancer specific survival (Hogberg et al., 2010).

1.3.10.2.4 Endocrine therapy

Current gynaecological oncology practice guide does not recommend progestins as adjuvant treatment of EC. Although this recommendation is based on pooled results of 11 clinical trials which are considered a high level of evidence (Martin-Hirsch et al., 2011), several points may need further reconsideration:

1. This meta-analysis considered progesterone only in the form of medroxyprogesterone (MPA) or megestrol acetate (MA) however recent

studies have shown that combination with other hormone therapy for instance tamoxifen improve survival in 33% of advanced/recurrent disease.

2. The status of steroid receptors has not been considered which could bias the study.
3. The groups are heterogeneous with different histological sub-type and staging systems, some of the studies were done in the early 1970s before the FIGO staging system was used.

Taking these factors together and bearing in mind the high evidence of continuous combined pills protective effect against EC it would be reasonable to reinvestigate both progesterone and tamoxifen as adjuvant therapy by an adequately powered prospective study.

1.3.10.2.5 Targeted therapy

Promising emerging data for several molecularly targeted agents reported 24% response to mTOR inhibitor (Ridaforolimus) in chemotherapy naïve patients; whereas patients with previous treatment showed only a 4% response rate with disease stabilisation in 46% (Oza et al., 2011; Colombo et al., 2013). Work on this targeted therapy is to be continued aiming to identify factors that can predict response and select patients most likely to benefit from mTOR inhibitor therapy.

1.3.10.3 Nonsurgical treatment

In addition to fertility sparing, surgery is not feasible in 5% – 10% of patients either because the disease is unresectable or due to medical contraindications (Colombo et al., 2013).

1.3.10.3.1 Radiotherapy

External radiation therapy with or without intra-cavity brachytherapy to the uterus and vagina can be used as primary line therapy when surgery is not feasible. 90%

of stage I patients achieved two-year local control rates. Image-guided brachytherapy may improve outcomes (Gill et al., 2014).

Radiotherapy with curative intent is indicated in patients with isolated vaginal relapse. Furthermore, radiotherapy can be effectively used to palliate symptoms such as bleeding, bone metastases or painful nodal recurrence (Colombo et al., 2015).

1.3.10.3.2 Chemotherapy

Metastatic EC shows a response rate to single dose cytotoxic agents of up to 40% in chemotherapy-naïve patients. Among those, platinum compounds, anthracyclines, and taxanes are most commonly used alone and in combination (Humber et al., 2007).

1.3.10.3.3 Combined chemo-radiotherapy

For vaginal or pelvic nodal recurrence, chemotherapy with radiotherapy could be considered in patients with high risk features for systemic relapse (Colombo et al., 2015).

1.3.10.3.4 Endocrine therapy

Fertility sparing: A conservative management approach could be considered in patients with a histological diagnosis of grade 1 EC in the form of MPA; 400– 600 mg/day or MA; 160–320 mg/day (Rodolakis et al., 2015). Some studies have shown a role for LNG-IUD as well. Preliminary data using gonadotrophic releasing hormone (GnRH) analogues depicted similar remission and recurrence rates to progestogens (Minig et al., 2011). However, since these hormonal agents are also contraceptive, a level of controversy exists regarding the suitability of these agents, since women will require abandoning these treatments to allow conception at a later date.

Relapsed disease: The front line for recurrent or persistent endometrioid histology only (Colombo et al., 2013) and involves mainly the use of progestogen agents. Oestrogen antagonist and aromatase inhibitors are used after starting progesterone. The overall response to sequential progestin and tamoxifen is 33% (Whitney et al., 2004).

1.4 Hypotheses and specific aims of this thesis

The work included in this thesis is aimed to fill the identified gaps that exist in the current literature regarding the role of AR, androgens and MIPs in EC. My aim was to test three main hypotheses;

Hypothesis 1. The expression of AR in human endometrium is dependent on the hormone milieu and is altered with the malignant transformation of the human endometrium and the AR expression profile will have a prognostic value for EC.

To test this hypothesis, I have

Examined three paraffin tissue section cohorts containing normal (pre and post-menopausal), hyperplastic (with and without cytological atypia), and neoplastic human endometrial (all subtypes) samples, for the expression of AR and the other steroid receptors

I have;

- I. Quantified the immunoexpression of AR in both endometrial epithelial and stromal compartments using an optimised and validated quick score system for endometrial steroid receptor expression.
- II. Compared AR protein immunoexpression with the expression of ER α , ER β , and PR and proliferation index Ki67.
- III. Investigated the prognostic value of AR on the survival of EC patients and its utility in predicting outcome.
- IV. Examined the alterations in transcript levels of AR and other steroid receptors and compared that with the protein expression profile.

Hypothesis 2. MIPs are differentially expressed in EC and can predict unfavourable EC outcome. AGR2 protein is secreted by EC cells.

To test this hypothesis, I have:

- I. Examined tissue samples from the three fore mentioned paraffin sections cohorts for immunoexpression of S100A4, S100P and AGR2 protein and quantified expression to compare between different cohorts.
- II. Investigated the prognostic value of the MIPs for predicting the outcome of EC patients.
- III. Examined the alteration in transcript levels and compared that with the protein expression profile.
- IV. Examined AGR2 protein level in serum samples, and uterine washing collected from EC patients before their primary surgery using protein binding assay (ELISA).

Hypothesis 3. Androgens via the AR reduce proliferation of human endometrial cancer cells and alter the expression of other steroid receptors and MIPs.

To test this hypothesis, I have:

- I. Used a non-aromatized, potent AR agonist, dihydrotestosterone (DHT), in the hormone responsive Ishikawa EC cell line model system to elucidate the role of AR in EC cell proliferation compared with the effect of oestrogen.
- II. Evaluated the effect of DHT on the expression of steroid receptors and MIPs in Ishikawa cells when co-cultured with stromal cells, simulating *in vivo* environment.

Chapter Two

General Materials and

Methods

2.1 Ethics

. The study was approved by Liverpool, Lancashire and Cambridge Adult Research Ethics Committee (LREC 09/H1005/55, 11/H1005/4 and CREC 10/H0308/75). Tissue with/ without blood samples were collected from patients who attended Liverpool Women's hospital (LWH) and Lancashire Teaching Hospitals Trusts from 2009-2014 after patients gave written informed consent.

2.2 Study groups

2.2.1 Endometrial cancer groups

A total of 100 histologically confirmed endometrial epithelial carcinoma samples; 60 of which were pipelles collected from patients who underwent staging operations at the LWH, the rest were paraffin blocks retrieved from the Histopathology Department archive at the Royal Hospital in Liverpool, or Lancaster Teaching Hospital in the period between 2009 and 2014. The histological type and grade of EC specimens were assigned by experienced gynaecological pathologists according to the International Federation of Gynaecology and Obstetrics (FIGO) and further detailed in 2.4.3. Metastatic lesions from 16 ECs were retrieved from Pathology Department of Lancaster Teaching Hospital archive (3 lymph node, 7 soft tissue, 3 parametrium, 3 omentum). Clinicopathological and demographic details of 97 patients were retrieved by reviewing the hospital notes and clinical databases (Table 2.1). Complete clinical information was not available for three patients and BMI was only available for 65 patients. None of the patients received hormonal treatment, chemotherapy or pelvic radiation prior to surgery. The standard primary treatment for EC at LWH and Lancaster Hospital is surgery. Depending on tumour stage and medical fitness of the patient surgical operation can be total hysterectomy, salpingo-oophorectomy, and/or pelvic and/or para-aortic lymphadenectomy with peritoneal washing cytology. Omentectomy is performed routinely for serous and clear cell carcinomas.

Table 2.1 Clinicopathological parameters of endometrial cancer cohort.

Parameter	N(%)
Tumour grade	
LGEC	50 (50)
HGEC	50 (50)
Histologic type	
Grade1	30 (30)
Grade2	20 (20)
Grade3	15 (15)
Serous	8 (8)
Clear cell	12 (12)
carcinosarcoma	15 (15)
Lymphovascular space invasion	
Negative	58 (59)
positive	40 (41)
Myometrial invasion	
<50%	57 (59)
≥ 50%	40 (41)
FIGO stage	
I	49 (50)
II	12 (12)
III	32 (33)
IV	5 (5)
Lymph node metastasis	
Negative	8 (8)
Positive	12 (12)
Not resected	77 (80)
Cervical stromal invasion	
Negative	70 (72)
positive	27 (28)
Extrauterine invasion	
Negative	65 (34.8)
positive	31 (16.6)

Abbreviations: Low grade cancers, LGEC; high grade cancers, HGEC.

2.2.2 Endometrial hyperplasia groups

As Table 2.2 shows, a total of 14 hyperplastic endometrial biopsies, 4 with no atypia (EHNA) and 10 with atypia (EHA) were collected from patients undergoing hysterectomy for previous histological evidence of hyperplasia with ongoing symptoms such as irregular or heavy menstrual bleeding at LWH or paraffin blocks of hyperplastic changes adjacent to EC retrieved from the Histopathology Department archive at the Royal Hospital in Liverpool. None of the included samples received hormonal therapy for three months prior to surgery.

Table 2.2 study groups.

Study groups	N
Proliferative phase	16
Postmenopausal	15
Endometrial hyperplasia	14
<i>No atypia</i>	4
<i>With atypia</i>	10
Endometrial cancer	100
LGEC	50
HGEC	50
Metastatic lesions	16

Abbreviations: Low grade cancers, LGEC; high grade cancers, HGEC

2.2.3 Control groups

Normal endometrial tissue specimens were obtained from patients undergoing hysterectomy for benign gynaecological pathologies unrelated to endometrial diseases such as prolapse and sterilisation. Exclusion/inclusion criteria are listed in Table 2.3. Two control groups were included in this study:

1. Postmenopausal control: as EC is a disease mostly affecting the PM aged women; 15 normal endometrial tissues from this age group were incorporated.
2. Proliferative phase control (PP): as EC is a proliferative disease, normal endometrial tissue from PP (n=15) were included as a second control group.

Table 2.3 Inclusion and exclusion criteria.

Study group	Inclusion criteria	Exclusion criteria
Postmenopausal	<ol style="list-style-type: none"> 1. Women should have amenorrhea for at least 12 consecutive months 2. Hysterectomy for non endometrial causes 	<ol style="list-style-type: none"> 1. Premature menopause <40 years 2. Exogenous hormone therapy in the past three months. 3. Endometrium with proliferative activity. 4. Any endometrial pathology
Proliferative phase	<ol style="list-style-type: none"> 1. Women with regular menstrual cycle. 2. Hysterectomy for non endometrial causes. 	<ol style="list-style-type: none"> 1. Exogenous hormone therapy in the past three months. 2. Irregular menstrual cycle 3. Any endometrial pathology

2.3 Collection of human samples

2.3.1 Endometrial tissue

Endometrial biopsies were collected by trained members of the research team or the operating surgeon in the theatre. Full thickness biopsies from normal pre or PM endometrium were obtained by cutting a thin slice of endometrium attached to underlying myometrium straight after opening the anterior aspect of the uterus in the coronal plane. To avoid interference with pathological diagnosis and staging, samples from EC were obtained by introducing a pipelle (Laboratoire C.C.D., Paris, France) into the endometrial cavity. After gentle scraping, the inner piston

of the pipelle was withdrawn allowing malignant tissue to be sucked into the pipelle under the effect of vacuum.

Collected samples are split into 2-3 containers depending on the size of the sample,

1. 15 mL neutral buffered formalin (NBF; Sigma-Aldrich, Poole, UK) to fix and preserve tissues for subsequent histology processing.
2. 15 mL Dulbecco's Modified Eagle Medium (DMEMF12) culture medium (Sigma, Dorset, UK) for subsequent stromal isolation or explant culturing.
3. 0.5 mL RNA later (Sigma, Dorset, UK) which rapidly permeates tissues to stabilise and protect cellular RNA for subsequent extraction.

2.3.2 Serum

Blood was collected from women who were included in the study by an anaesthetist immediately prior to the operation whilst obtaining venous access (to ensure no extra venepuncture is necessary). Five mL blood in a 10 mL EDTA free tube was allowed to clot at room temperature for 30 min and centrifuged for 10 min at 3000 g. Amber clear serum was subsequently transferred to 5mL Bijou tubes under aseptic conditions, within a class II hood, mixed and aliquoted in labelled cryo-vials and stored in -80 freezers.

2.3.3 Uterine wash

The procedure carried out by DH in the theatre prior to starting the assigned operation (hysterectomy or hysteroscopy). In the lithotomy position, after cleaning the vaginal and cervix with physiological saline solution a Simm's speculum was inserted into the vagina to expose the cervix., a quills catheter attached to a 10mL syringe was inserted into the uterine cavity and 10 mL fresh normal saline was gradually flushed into the uterine cavity. After a minute, the fluid was recovered by gentle suction by withdrawing the attached syringe plunger and transferred into

a universal container. The sample was subsequently transferred to the lab on ice where it was centrifuged for 5 min at 3000 rpm. The supernatant was then aliquoted into 1.5 mL Eppendorf tubes and stored in a -80 freezer.

2.4 Tissue preparation and characterisation

2.4.1 Formalin fixed paraffin embedded samples preparations

2.4.1.1 Tissue processing

All samples were preserved in 10% neutral buffered formalin (NBF) directly after collection to avoid autolysis. After 1-5 days in NBF, the samples were ready for processing which was undertaken in an automated processing machine (ShandonHistoCentre3, Cheshire, UK).

Inside the processor, the tissue specimens were first dehydrated by conveying them automatically through increasing concentrations of ethanol before immersing the cassettes in the clearing agent. The clearing agent has the essential function of replacing the dehydrant with a substance that is miscible with the paraffin. Xylene, which is the commonest clearing agent, was used in our lab. Finally, the samples were infiltrated with the embedding agent, paraffin.

2.4.1.2 Tissue embedding

The tissue cassettes produced from the automated processor were manually put into block moulds using an embedding platform (ShandonHistoCentre3, Cheshire, UK). Pre-warmed tissue biopsies were picked out of the cassettes, aligned properly in a pre-warmed mould before molten paraffin was poured over them. After labelling the blocks, they were left on the cold plate to cool. The blocks were kept in the freezer overnight or for at least 30 minutes before sectioning.

2.4.1.3 Sectioning

Once the block is ready and cooled 3-4um sections were cut using the Micron rotary microtome (Microm HM335, Thame, UK). A ribbon of approximately six

sections was cut at a time, each section was floated in a pre-warmed water bath separately, left for few minutes to remove wrinkles and then collected on 3-Aminopropyltriethoxysilane (APES) coated glass slides and left to dry at room temperature overnight.

2.4.2 Cycle staging

The premenopausal noncancerous endometrial tissue specimens were phase typed according to the patients last menstrual period (LMP) and histological criteria (Noyes et al., 1950; Dallenbach-Hellweg et al., 2010) by examination of the haematoxylin and eosin (H&E) stained endometrial biopsy. This method was further confirmed by reviewing the official pathological reports.

2.4.3 Endometrial cancer confirmation and classification

The histological type and grade of EC specimens were assigned by experienced gynaecological pathologists according to FIGO (Zaino et al., 1995). All collected samples were reviewed for adequacy by identification of representative malignant tissue in H&E stained sections before inclusion in the study. Sections that were morphologically inconsistent with pathology reports were reviewed with a consultant pathologist (YM) and re-assigned based on the agreed type.

Different EC classifications have been discussed in 1.3.3. For descriptive and analytical purposes, EC has been categorised in this study into (Voss et al., 2012) :

- Low grade tumours (LGEC): This group include grade 1 and grade 2 endometrioid EC which have favourable outcomes.
- High grade tumours (HGEC): This group include grade 3 endometrioid, serous, clear cell carcinomas and carcinosarcomas.

2.5 Cell culture

Hormone modulation was carried out *in vitro* by treating established Ishikawa cell lines in monoculture or co-cultured with human endometrial stromal cells with 17 β oestradiol (E2), 5 α dihydrotestosterone (DHT) individually or in combination and with their antagonists. All cell culturing procedures were performed under sterile conditions in BioMat² Class II hoods (CAS, Manchester, UK). Cell culture reagents and steroid hormones were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise specified.

2.5.1 Monoculture

2.5.1.1 Ishikawa cell line culturing

The cell line Ishikawa (ISK) was established from a well-differentiated grade 1 human endometrial adenocarcinoma (Nishida et al., 1985). These cells have been shown to express all steroid receptors (Hata and Kuramoto, 1992; Lovely et al., 2000). ISK cells were obtained from Public Health England (Salisbury, UK) and gene-profile was confirmed by Short Tandem Repeat profiling (STR).

These cells were maintained and grown in a 10cm culture dish containing DMEM/F12, 10%FBS (BioSera, UK) supplemented with L-glutamine (2mM final) and the antibiotic, penicillin/streptomycin (1/500 in media; InvivoGen). Cells were incubated at 37°C under 5% CO₂ in a humidified atmosphere. The medium was changed 24 h after seeding and renewed at intervals of 48 h until cell culture achieved subconfluence. Cells were passaged at 1:5 dilutions using trypsin, the 3rd to 12th passages were used for the experiments.

The cells were prepared for steroid treatment by preconditioning in DMEM/F12 (phenol red-free, Life Technologies, UK) supplemented with antibiotic and 2% charcoal-stripped FBS (CS-FBS) for 48 hours prior to the experiment.

2.5.1.2 Immortalised human endometrial stromal cells culturing (hESC)

The hESC line was established by immortalisation of normal human endometrial stromal cells by retroviral transfection with human telomerase reverse transcriptase (Krikun et al. 2004). The karyotype, phenotype, and morphology of this cell line have been shown to be similar to the primary parent cell. (Krikun et al., 2004). This cell line was kindly donated by Graciela Krikun of Yale University, USA.

The hESC were grown in DMEM/F12 (phenol red -free, Life Technologies, UK) supplemented with antibiotic and 10% FBS, L-glutamate and antibiotic and incubated at 37°C under 5% CO₂ in the air. Cells were prepared for steroid treatment in the same way ISK cells were preconditioned (see 2.5.1.1)

2.5.1.3 Primary stromal cells isolating and culturing

Pipelle tissue biopsies that had been placed in collection media (see 2.3.1) were transferred to a 100mm Petri dish along with 1mL of DMEM to maintain tissue moisture. A surgical blade was used to cut the endometrial tissue to a fine mince consistency. Following this, the tissue was transferred to a 30mL universal tube using a 1mL pastette and the Petri dish was rinsed with DMEM to ensure all remaining tissue was collected. The tube was centrifuged at 500g for 5 minutes to collect the cells in pellet form. The cell pellet was resuspended in 4mL of DMEM, 500µl collagenase (20mg/mL in PBS; Life Technologies, UK), 500µl dispase (10mg/mL in PBS; Life Technologies, UK) and 100µl DNase (4mg/mL in PBS; Roche Diagnostics, UK). The suspension was incubated for 90 minutes in a shaking water bath at 37°C to allow digestion to single cells. The digest was triturated periodically to dissociate digested tissue. Following this, the digest was filtered through a 40µm cell strainer (Fisher Scientific, Loughborough, UK) into a 50mL tube and the filter was washed thoroughly with 30-40mL of DMEM. Epithelial glands, which were too big to pass through the filter were retained. The stromal cells and contaminating red blood cells which passed through the filter were collected by centrifugation as before.

Red blood cells were lysed by incubating the cell suspension with 15mL of hypertonic solution for 3 minutes. The reaction was stopped by adding PBS to 50mL and the cells were collected by centrifugation at 500g, for 5min. Selective adherence to tissue culture plates was used to minimise epithelial cell contamination of the stromal cell fraction. The crude fraction of stromal cells was subsequently plated in 10 mL dish of primary cell media (DMEM/F12 + primocin+10% FBS) and incubated for 20 minutes allowing stromal cells to settle down (#1). The medium containing cells that had not attached was then aspirated and plated in a new culture dish, and 10mL of fresh primary media was added to #1. Both plates were incubated at 37°C under 5% CO₂. Cells were fed every 3-4 days and not used beyond passage three.

2.5.2 Co-culture

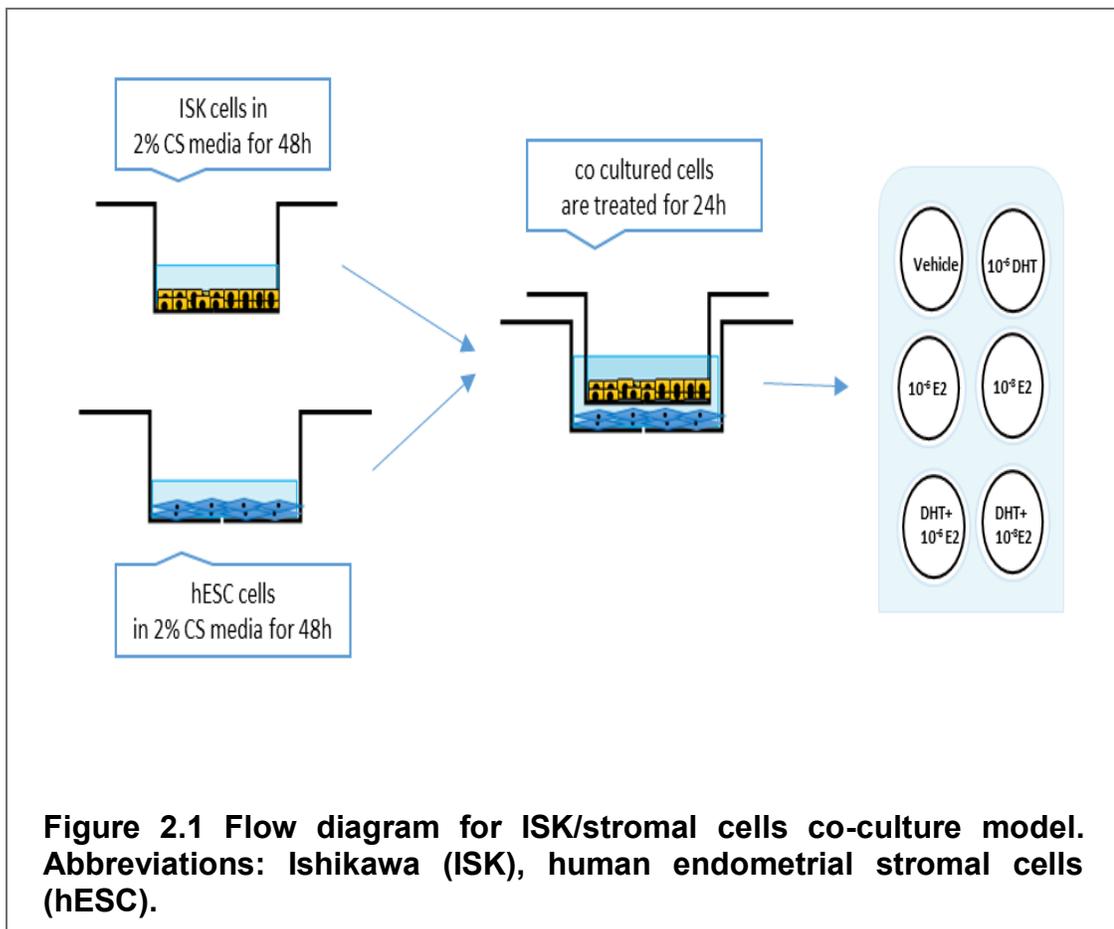
To create a condition which preserves stromal/epithelial cross-talk and maintains cell purity for downstream experiments, ISK epithelial cells were cultured with either primary or hESC in a transwell system (Corning, USA) as shown in Figure 2.1. Briefly, ISK cells were seeded in transwell inserts with 0.4 mm pores at a density of 3×10^5 cell/insert and cultured in DMEM/F12 with 2% CS-FBS for 48h. The total cell numbers were determined using hemocytometer. Similarly, hESC or primary stromal cells were seeded in 6 well plates at a density of $\sim 15 \times 10^4$ and cultured in DMEM/F12 with 2% CS-FBS. After 48h, culture media in bases and inserts was replaced with 2.6 and 1.5mL DMEM/F12 with 2% CS FBS \pm treatment respectively. Subsequently, inserts were placed on the top of bases as shown in Figure 2.1 and ISK/stromal co-culture was incubated for 1-3 days. At the indicated time points, cells were harvested by trypsin, pelleted and stored for downstream experiments at -80°C.

2.5.3 Explant culture

Explant culture is a biological model where tissue composition, architecture, and communication between cells are maintained. This model was used to investigate

the spatial and temporal expression of steroid receptors in response to hormone modulation.

The endometrial biopsies were separated from the medium and transferred to a Petri dish, rinsed in PBS and cleared of blood and mucus. Subsequently, the tissue was cut into pieces of 1–2 mm³ and preincubated in DMEM/ F-12 medium with 2% CS-FBS for 30 minutes until hormonal treatment was ready.



For the main incubation, the explants (3–5 pieces) were placed in a 6-well plate and cultured in 3 mL of fresh 2%CS FBS medium with or without treatment for 24h. Explant cultures were subsequently fixed in 10% NBF for 24h before processing.

2.5.4 Steroid hormone treatment

The steroid hormones were added from 1000-fold concentrated stocks made in absolute methanol to the desired concentration of DHT (D-073-1mL, Sigma) E2; (E8875-250mg, Sigma) individually or in combination and with an antagonist such as fulvestrant (ICI 182,780, Tocris Bioscience, UK) or bicalutamide (CDX, B906, Sigma). The time of exposure in each case was approximately 1-3 days. In some experiments, cells were also cultured with epidermal growth factor (EGF; 10ng/mL) as a positive control for cell proliferation.

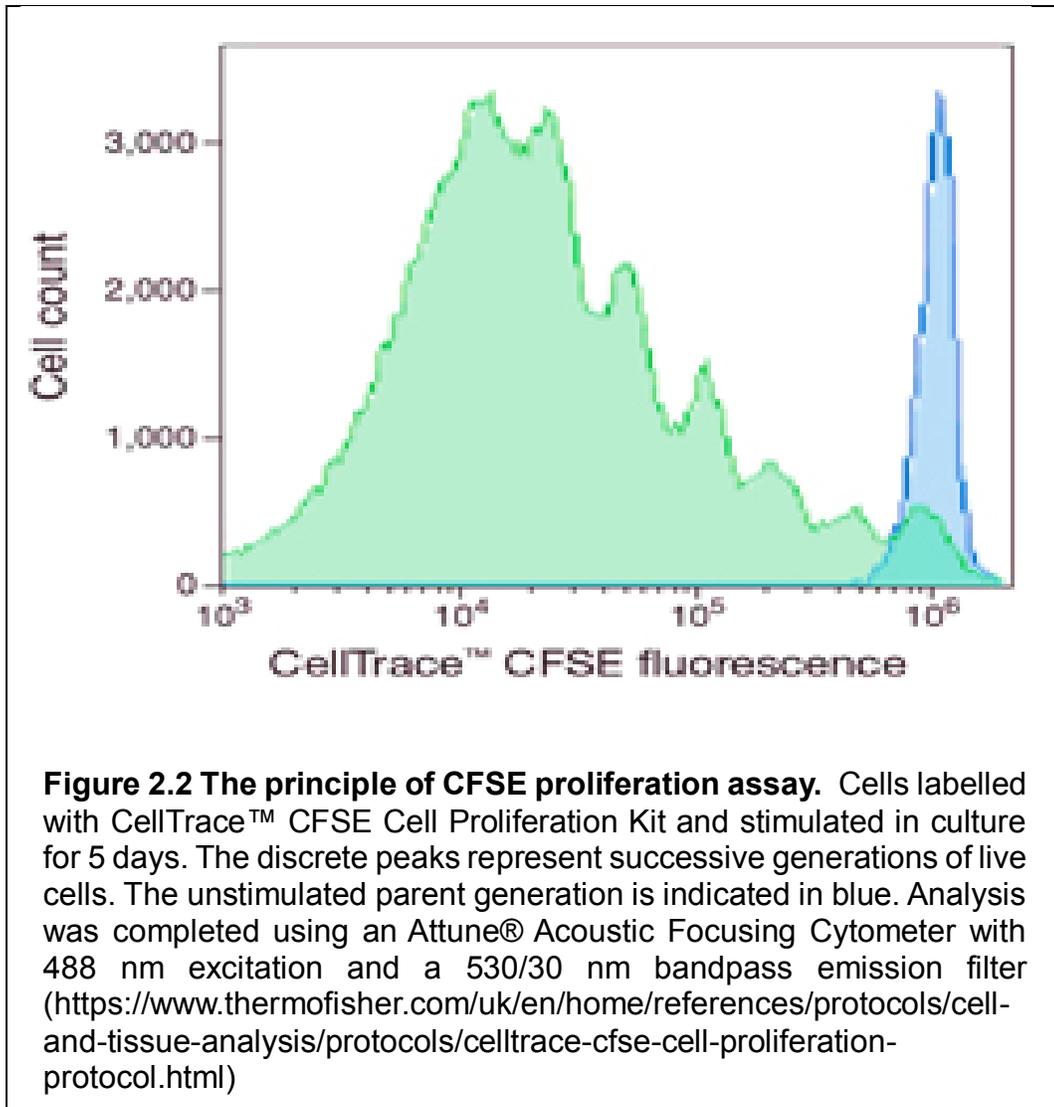
2.6 CFSE Proliferation assay

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) is an intracellular fluorescent stain with a fluorochrome wavelength in the range of the FITC stain. It binds to intracellular molecules especially lysine residues by forming covalent bonds with the privilege to be retained in cell for very long time. CFSE has been used extensively to measure cellular proliferation by flow cytometry. CFSE-labelled cells proliferation will lead to spread of the CFSE stain on to a greater number of cells (daughter cells) which appear on the histogram of the FACS as a shift of the curve to the left with diminished peak (Figure 2.2).

2.6.1 Cell labelling:

Preconditioned ISK cells were trypsinised and washed twice with PBS then spun down. Meanwhile, 5mM CellTrace CFSE (C34554, Thermo Fisher Scientific, UK) stock solution was freshly prepared by dissolving the contents of one vial of CFSE (2 μ L) with 18 μ L of Component B (DMSO). This was diluted in PBS to the desired working concentration (1 μ M) with enough fluorescence and less harm to the ISK cells. ISK cells were then resuspended with CFSC working solution at 1 \times 10⁶ cells/mL and incubated for 15 minutes at 37°C in darkness with shaking several times every 3 minutes. Five times the original staining volume of culture medium (containing at least 1% protein) was then added and incubated for 15 min to stabilise the CFSE staining and remove any free dye remaining in the solution. The cells were then pelleted and resuspended in 2% CS-FBS and incubated

overnight. The next day cells were treated with steroid hormones individually or in combination and with steroid receptor antagonists for 3 days.



2.6.2 Flow cytometry:

Flow cytometry was performed at the Department of Children's Health/ Alder Hey Hospital. Cells from each condition were trypsinised and centrifuged at 500 g for 5 min then resuspended in 1.5-2 mL of cultivating media. 1mL of cell suspension was transferred to a FACS tube and left on ice for 10 min and protected from light.

Fluorescently labelled single-cell suspensions flow past laser excitation of 488 nm, in a liquid phase. The stream of cells is broken into individual droplets containing single cells. As the cells pass through the beam they cause the light to scatter, this is analysed by detectors which can assess the cell structure (FC). Data were analysed using public domain Cyflogic software version 1.2.1 (Perttu Terho, Mika Korkeamaki, CyFlo Ltd).

2.7 Protein expression and quantification

2.7.1 Immunohistochemistry

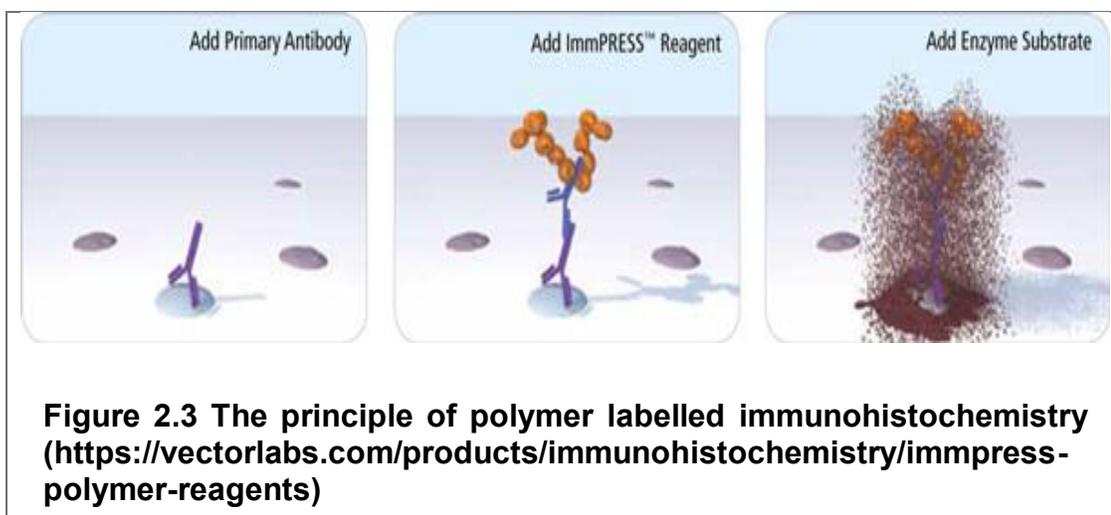
The temporal and spatial expression of steroid receptors and MIPs proteins were investigated by immunohistochemistry (IHC). Figure 2.3 outlines the principles of IHC.

2.7.1.1 IHC protocol:

Preheated sections were initially deparaffinised in xylene and rehydrated through graded alcohol. To enhance immunostaining, these sections were heated in a pressurised chamber for 1-2 minutes with an appropriate buffer optimised according to antibody (Ab) affinity. The sections were then incubated in 0.3% H₂O₂ /TBS (Sigma, Aldrich) bath for 10 min to block endogenous peroxidase.

In a humidified chamber, sections were incubated with the specified Ab directly after blocking unspecific binding with horse serum block for 20 min. A drop of Vector ImmPRESS labeled polymer-HRP matching the primary antibody (Vector Laboratories, Peterborough, UK) was then added to each sample and incubated for 30 min followed by DAB-substrate for 10 min then immersion in distilled water for 5 min.

Each step in the staining protocol has been carried out at room temperature unless stated otherwise. At the end of each step, sections were rinsed in Tris-buffered saline (TBS) in order to hold the reaction and remove any unbounded material that would interfere with subsequent steps.



Finally, the sections were counterstained with Gill haematoxylin (Thermo Scientific, Runcorn, UK) and mounted in synthetic resin after being dehydrated through graded alcohol.

A negative control was included in each run to assess the specificity of the primary Ab. For the negative control the primary Ab was substituted with a matched type and concentration of immunoglobulin. An internal positive control was also included in each run to identify any staining variation over runs.

2.7.1.2 IHC analysis:

Hscore: Stained sections were scored using a light microscope at x400 magnification. For each sample, a representative first field of vision was selected randomly and photographed using (Nikon, Tokyo, Japan), followed by nine consecutive fields. This systematic at random sampling minimises the bias of selecting positively stained foci. The fraction of negative (score 0), weakly positive (score 1), moderately positive (score 2) and strongly positive (score 3) were counted using Image J software. The intensity and average percentage score of ten fields were then multiplied and scores summed to give the final score.

Quick score was obtained by scanning the whole section and estimating the percent of stained proportions in each of four intensities. The proportion bands

were determined for each target protein; this will be detailed in related chapters along with the optimisation. The total score out of 12 was calculated by multiplying the intensity score 0, 1, 2, 3 by the proportion score.

The proportion of positive staining was used to determine the proliferation index (Ki67) whereby, sections were scanned under the microscope and the positive staining of any intensity was estimated as a percent.

To identify the carcinomatous epithelial cells in carcinosarcoma (CS), a serial section for each CS sample was immunostained with pan-cytokeratin (Panck). Only Panck positive cells were scored for the target protein.

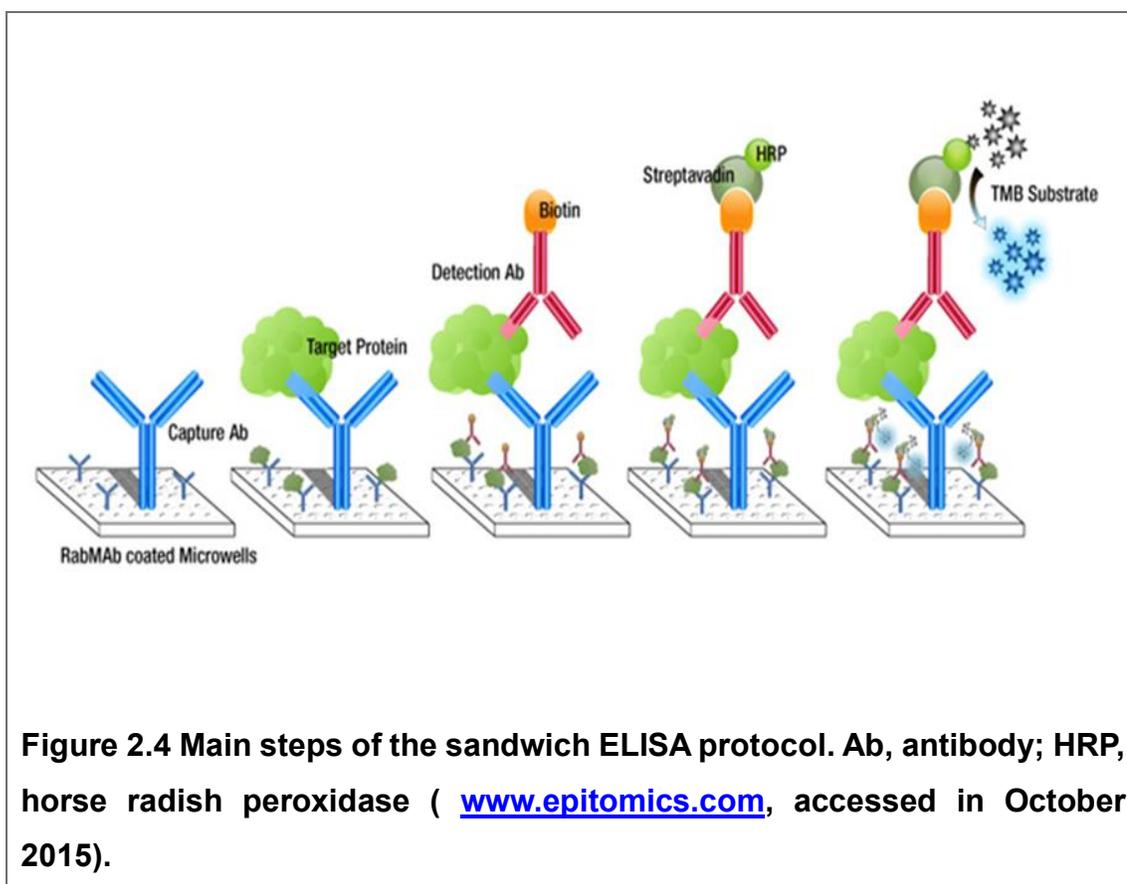
Consistency and reproducibility of immunoscores were assessed by revisiting randomly selected and blinded sections by myself (intraobserver) and an independent observer DH, AL or EB (interobserver). The high agreement was obtained in both the Inter and intraobserver's scoring. Conflicting scores were reviewed and the agreed score was finally considered.

2.7.2 Enzyme-linked immunosorbent assay (ELISA) for AGR2

ELISA is a very common technique used to quantify proteins in tissues, cell cultures or fluids, whereby the antibody or the antigen adsorbs to a solid surface and still participates in specific high affinity binding, facilitating the separation of bound and free antigens. Sandwich (capture) ELISA is the format of choice when the antigen is being detected.

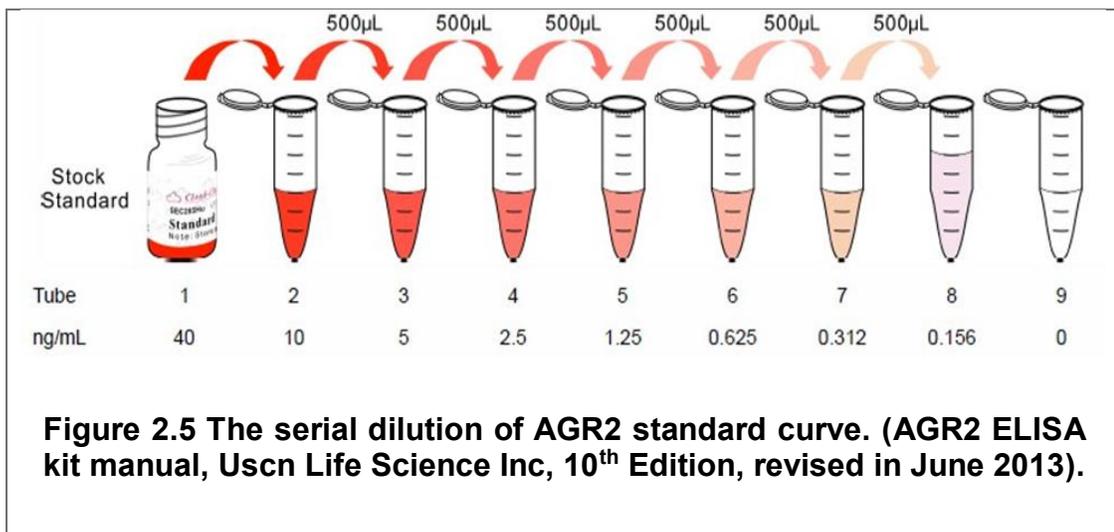
AGR2 level in the serum and uterine washes from normal PM and EC patients was detected using commercially available AGR2 ELISA kit (SEC285Hu, Uscn Life Science Inc., China). Following the manufacturer's instructions, a sandwich ELISA protocol was carried out as outlined in **Figure 2.4**. Briefly, the standard was reconstituted with 1 mL (40ng/mL) of standard diluent and kept for 10min at room temp. Seven serial double fold dilutions were subsequently prepared as shown in

Figure 2.5. A solid phase, ready to use a 96- well strip plate pre-coated with the capturing Ab specific to AGR2 was provided.



Standards, unknown samples or blanks were added to designated wells, covered with plate sealer and incubated for 2h at 37°C. After that, the liquid was removed from the wells and 100µL of Reagent A (biotinylated Ab specific to AGR2) was added to each well directly without washing, sealed and incubated for 1 hour at 37 °C. Excess reactants were diluted by washing the wells with 350µL of Washing Solution for three times followed by snapping onto adsorbent paper to remove any remaining liquid and bubbles. Subsequently, 100µL of Detection Reagent B (Avidin – Horseradish peroxidase) was added to each well, covered and incubated for 30 min at 37°C followed by 5 washes. Next, 90µL of tetramethylbenzidine (TMB) Substrate Solution was added to each well, sealed and incubated in the dark for 10-20 min until a blue colour developed. All incubation steps were carried out on a shaking platform (Dynatech Varishaker-Incubator). The enzyme-

substrate reaction was terminated by the addition of 50 μ L of Stop Solution (sulphuric acid) and the colour change was measured spectrophotometrically (Multiskan Ascent ELISA, Thermo Electron Corporation, Hampshire, UK) at a wavelength of 450nm. Following the fact that the passage of light through a coloured solution is directly related to the amount of colour present, the concentration of the AGR2 was measured by comparing the optical density of the samples and the standard.



2.8 Messenger RNA (mRNA) expression and quantification

2.8.1 RNA isolation

RNA isolation is the most critical step that determines the reliability of the downstream outcome. TRIzol/chloroform extraction is based on powerful guanidinium isothiocyanate protein denaturation and RNase inactivation followed by an acid phenol/chloroform phase separation (Chomczynski and Sacchi, 1986). Guanidine isothiocyanate is a chaotropic salt; it destabilises hydrogen bonds, van der Waals forces, and hydrophobic interactions. Thereby, proteins are destabilised, including nucleases, and the association of nucleic acids with water

is disrupted setting up the conditions for the transfer to silica. Silica-cartridge purification of RNA is the recommended RNA purification method in the gene expression industry. RNA in the lysed sample is bound to the clear silica-based membrane in a spin cartridge whereby chaotropic salts enhance and influence nucleic acid binding to silica as well as alcohol. After centrifugation of the lysate, extracted RNA should be bound to the membrane and the impurities, protein, and polysaccharides, should have passed through; however, residual proteins and salts might remain attached that require a further washing step. There are typically two washes; the first wash will often have a low amount of chaotropic salt to remove the protein and coloured contaminants, the second is an ethanol wash to remove the salts.

The final step in the RNA extraction protocol is the release of pure RNA from the silica. RNA dissolves readily in water and is fine at a slightly acidic pH and so water is the preferred diluent.

2.8.1.1 Extraction

Total RNA was extracted from frozen EC tissue specimens utilizing a TRIzol Plus RNA Purification Kit (Invitrogen Ltd., Paisley, United Kingdom) following the manufacturer's protocol.

Briefly, cells and RNA-later samples were homogenised in 1mL of TRIzol reagent. The resulting cell lysate was passed several times through a pipette tip to shear the DNA then incubated for 10 min at room temperature. Chloroform (0.2mL) (Sigma, Poole, UK) was added and mixed for 60 sec followed by incubation for 5 min at room temperature. Samples were then centrifugated at 12,000g for 30 min at 4°C. The upper aqueous phase containing RNA was transferred to a new RNase-free tube. An equal volume of 70% (v/v) ethanol was then added and vortexed.

2.8.1.2 Purification

Approximately 700µl of the RNA/ethanol solution was then loaded onto a PureLink RNA Mini Kit spin cartridge and centrifuged at 12,000g at room temperature for 15 min. The spin cartridge encompasses a silica-based membrane to which the RNA binds during purification. The flow through was discarded and the above step repeated until the whole sample was processed.

Lastly, the washing step was carried out by adding 700µl of wash buffer I and centrifuging at 12,000g for 15 secs at room temperature, followed by two washes and centrifuge spins with wash buffer II to dry the membrane with the attached RNA. The spin cartridge was then transferred to a new recovery tube where 30µl of RNase-free water was added to the centre of the spin cartridge and left for 1 min. This step was repeated twice, adding a total of 90µl of RNase-free water and centrifuged at 14,000g, for 1 min at 4°C. The purified RNA samples were stored in a -80°C freezer.

2.8.1.3 Quantification

RNA concentrations and purity were determined spectrophotometrically at (260/280 ratio) using a NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). A260/ A280 ratio of 1.8+ was considered as suitable for downstream reactions.

2.8.2 DNase treatment

To remove any contaminating genomic DNA before transcriptase reaction, 8µl of purified RNA was treated with 1µl (2 units) of DNase I enzyme (DNase I (#M0303), NEB, Hertfordshire, UK) in the presence of 1µl (10X) DNase I reaction buffer and incubated for 10 min at 42°C. 1µl of EGTA (Promega, Hampshire, UK) was added and incubated for 10 min at 75°C to stop the reaction.

2.8.3 RNA clean up

RNA with low purity, concentration or TRIzol carry over were further cleaned up using the MiniElute clean-up kit (Quaigene, Manchester, UK). Total RNA was first diluted with nuclease free (NF) H₂O and DNase treated as described earlier 2.8.2 to make up a 100µl final volume. Selective binding to the silica membrane was enhanced by mixing RNA with 250µl of Guanidine thiocyanate containing lysis buffer and 350µl absolute ethanol. RNA binds to the silica membrane, contaminants are efficiently washed away, and 12µl high-quality RNA is eluted in RNase-free water.

2.8.4 First strand DNA synthesis

To remove any secondary structure that may impede long cDNA synthesis 0.3 - 1µg of purified total RNA was first denatured at 70°C for 5 min in the presence of 2µl Oligo (dT) and RNAase free water to make up to 8µl then promptly cooled on ice to stop the reaction. Subsequently, 10µl of 2X reaction mix and 2µl AMV reverse transcriptase RT (NEB, Hertfordshire, UK) was added to make a total volume of 20µl. The mix was incubated at 42°C for 60 min for maximum yield and length then further heated to 80°C for 10 min to stop the reaction. The 1st strand cDNA was finally stored at -20°C.

2.8.5 Polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful molecular technology whereby specific sequences of the cDNA or DNA template can be amplified million folds using sequence specific oligonucleotides, heat stable DNA polymerase and thermal cycling. PCR theoretically amplifies DNA exponentially, doubling the number of target molecules with each amplification cycle as shown in **Figure 2.6**.

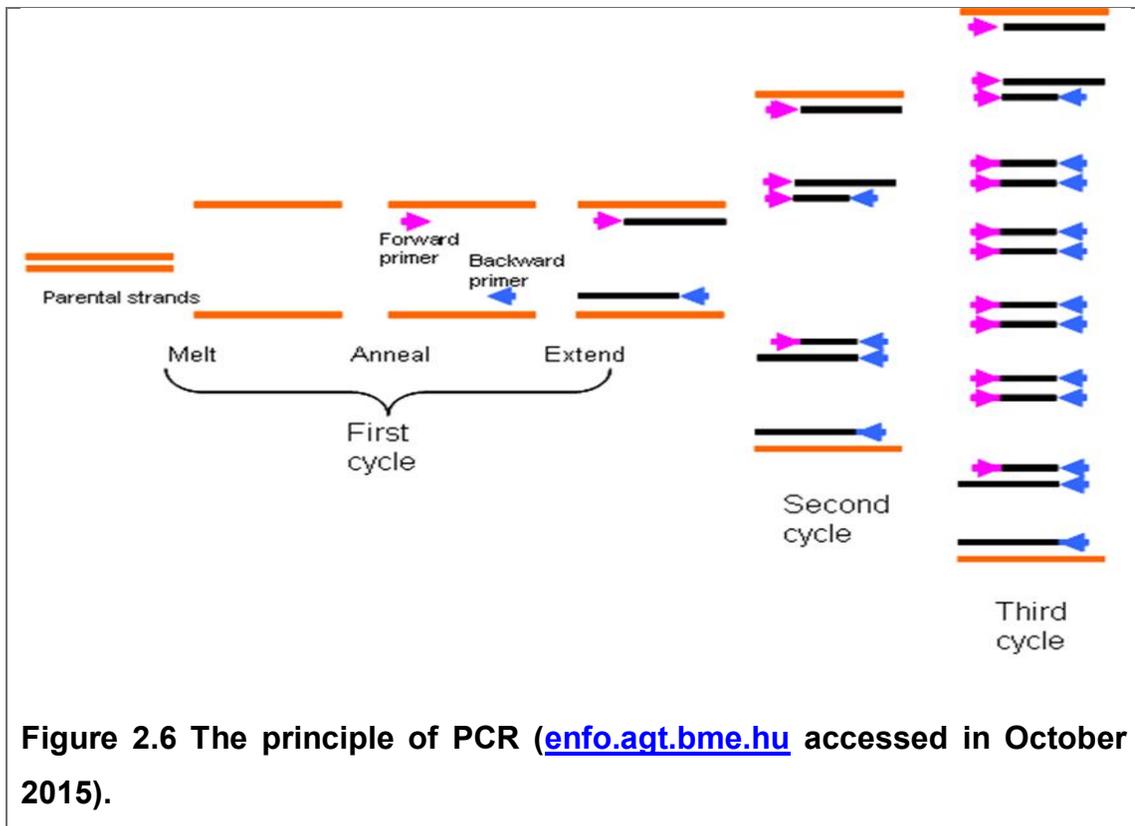


Figure 2.6 The principle of PCR (enfo.agt.bme.hu accessed in October 2015).

Thermal cycling is based on three major steps:

3. **Denaturation:** High temperature incubation is used to melt double strand DNA and loosen the secondary structure into single stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95 °C). The denaturation time can be increased if the template GC content is high.
4. **Annealing:** During annealing, complementary sequences have the opportunity to hybridize based on the calculated melting temperature (T_m) of the primers, typically 5 °C below T_m , the appropriate temperature is used.
5. **Extension:** At 70-72 °C, the activity of DNA polymerase is optimal, and primer extension occurs at a rate of up to 100 bases per second. When the amplicon is of small size, in a real-time reaction, this step can be combined with the annealing step using 60 °C.

2.8.5.1 Endpoint PCR

Detection and quantification of the amplified sequence are performed at the end of the reaction after the last PCR cycle and requires post amplification gel electrophoresis analysis.

PCR amplifications were performed in a final volume of 10 μ l. A dilution of 1:20 of the first cDNA was mixed with a primer mix made of 0.25 μ l of each forward 5' and reverse 3' oligoes in 0.5 PCR water. Hot Start Taq (2x) was added before placing the samples in Bio-Rad T100 Thermal Cycler (Bio-Rad, Hemel Hempstead, UK). To ensure proper mixing, vortexing and centrifuging was performed after each step. An initial denaturing step of 95°C for 2.5 min was followed by a primer specific number of cycles at 95°C for 30 secs, 45 secs annealing at 60°C and a final extension for 2.5 min at 72°C.

2.8.5.2 Real-time qPCR

Real-time PCR is a highly sensitive technique enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. The fluorescent dye SYBR Green binds all double stranded DNA molecules nonspecifically, emitting a fluorescent signal. Signal intensity increases with an increasing cycle number due to the accumulation of PCR product.

The PCR reaction constituted 5 μ l of 2x JumpStart Master Mix, 0.25 μ l forward (F) and reverse (R) primers at 250 nM (Sigma, Poole, UK), 0.5 -1 μ l of cDNA and nuclease free water up to a final volume of 10 μ l. The reaction was set following the conditions optimised for each target at 37-40 cycles. All samples and negative controls were amplified in duplicates, and the obtained mean value was then used for further analysis.

The efficiency and reproducibility of qPCR experiments were assessed through the generation of a standard curve for each target and reference gene. A dilution

series of cDNA spanned the concentration range expected for the experimental samples amplified. The results were then plotted with input nucleic acid quantity on the x-axis and cycle threshold (C_t) on the y-axis. The slope of the curve was used to determine the reaction efficiency which was within the accepted range (90%- 110%) for all primers used. Reproducibility of the experiment determined by the R^2 value of the standard curve was accepted at ≥ 0.996 .

2.8.6 Gel electrophoresis

After PCR, the products were resolved on a 1.6-2% agarose SYBR Safe gel. Agarose powder (1.6-2g) (Sigma, Poole, UK) was dissolved in 100mL TEA buffer (Thermo Fisher Scientific, Loughborough, UK) and heated in the microwave until completely clear. Having cooled to approximately 60°C, 6 μ l of SYBR Safe (Invitrogen, Paisley, UK) was added, mixed gently and poured into the electrophoresis tray where it took 30 min to solidify. Before loading the samples, electrophoresis buffer (1xTAE buffer) was used to cover the agarose gel by 1-2mm. A mixture of 4 μ l of PCR product and 1 μ l of loading buffer (NEB, Hertfordshire, UK) was loaded into the wells set in the gel. The gel was run at 80v for 45 min. Bands were visualised using ChemiDoc-ItTS2 Imaging Systems (UVP, California, United States).

2.8.7 Gene expression analysis

Relative quantification is considered the method of choice for gene expression studies. The expression level of a gene of interest is assayed for up or down regulation in normal and one or more experimental samples. Precise copy number for this technique is not required, instead, fold change between the sample and the control is the main focus.

The $\Delta\Delta C_t$ method has been used to compare the results from experimental samples with both a calibrator (untreated sample) and the geometric mean of normalisers (reference genes). For a gene of interest both the test sample and

the calibrator are adjusted in relation to the mean of the normalisers C_t from the same samples. The resulting $\Delta\Delta C_t$ value is incorporated to determine the fold difference in the expression.

$$\text{Fold differences} = 2^{-\Delta\Delta C_t}$$

$$\Delta C_{t \text{ sample}} - \Delta C_{t \text{ calibrator}} = \Delta\Delta C_t$$

$$C_{t \text{ target}} - C_{t \text{ reference}} = \Delta C_{t \text{ sample}}$$

$$C_{t \text{ target}} - C_{t \text{ reference}} = \Delta C_{t \text{ calibrator}}$$

2.9 Statistical analyses

Statistical differences between groups were calculated by non-parametric tests (Kruskal–Wallis/Dunn’s post hoc, Mann–Whitney U-test or Wilcoxon signed rank test) using Statistical Package for the Social Sciences (SPSS) version 21. Descriptive values were presented as median and range. The correlations between immuno-expression scores were examined with Spearman test and association between immuno-scores and the multiple clinicopathological parameters with Pearson Chi-square. Disease-free survival (DFS) was measured from the date of surgery to the date of EC recurrence or death from EC. Cancer-specific survival (CSS) was measured from the date of surgery to the date of death from EC; the observations were censored at death for causes other than EC; recurrences were ignored. Overall survival (OS) was calculated from the date of surgery to the date of death from any cause; recurrences were ignored. All observations were censored at loss to follow-up and at the last data update. For survival analysis, each parameter was categorized and survival curves were obtained using the Kaplan-Meier method. Cumulative proportions of survivors in the presence and absence of the marker under investigation were compared using Log rank test. The Cox proportional hazards regression model was used to identify the independent prognostic factors. Only variables with a $P < 0.05$ in the

univariate analysis were included in the multivariate model. $P < 0.05$ was considered significant.

Chapter three

The Prognostic Role of Androgen Receptor

3.1. Introduction

The endometrium is the main target organ for ovarian hormones and steroid hormones are implicated in carcinogenesis of the endometrium and other classical hormone responsive tissues such as breast and prostate. Endometrial cancer (EC) is generally a disease of the PM period that is defined by cessation of the cyclical production of ovarian hormones. Since these hormones exert their effect via their cognate receptors, in common with other hormone responsive cancers such as breast cancer (Tagnon, 1977; Thike et al., 2001), the hormone receptor status of ECs would be expected to play a role in predicting clinical outcome and guiding therapeutic choice (Zhang et al., 2015). Unlike breast cancer (Anonymous, 1994), steroid receptor status is not routinely reported for EC, yet increasingly clinical oncologists in the UK seek this information in high grade EC to make therapeutic decisions beyond standard surgery (Singh et al., 2007).

The expression of oestrogen receptor alpha (ER α), oestrogen receptor beta (ER β) and progesterone receptor (PR) in EC (at least endometrioid subtype) have been extensively studied; nevertheless, prognostic significance remains conflicting (Fujimoto et al., 2000; Jongen et al., 2009; Zannoni et al., 2013). Many of these studies have used different control tissues and scoring systems which make the collation of the available data difficult and may explain the seemingly contradictory results.

Defining the contribution of all steroid receptors, including the AR, which has not been described in detail in EC previously, to the initiation, progression, and prognosis of EC will improve the understanding of the hormonal changes that precede and potentially drive EC tumorigenesis.

3.1 Research questions

1. Could we develop a quick scoring system which can efficiently quantify all steroid receptors in both endometrial compartments (epithelial and stromal)?
2. In normal non-pathological conditions, how would the spatial and temporal expression pattern of AR change in the endometrium of PM compared with functionalis and basalis layers of the full thickness PP endometrium?
3. How would the expression profile of AR and other steroid receptors (ER α , ER β and PR) vary in different stages of endometrial carcinogenesis; EH, different EC histological sub-type and surgical stage and metastatic lesions? And would their expression correlate with proliferation marker and clinicopathological parameters of the patients?
4. Can AR predict tumour progression, cancer specific or overall death independently of other prognostic factors?

3.2 Methods

3.2.1 Patient population

Patient groups are detailed in Table 3.1. Eighty-five EC, 16 metastatic lesions (3 lymph node, 7 soft tissue, 3 parametrium, 3 omentum), 12 hyperplastic (4 without cytological atypia, EHNA; 8 with cytological atypia EHA) and 28 full thickness normal endometrial biopsies were collected from patients undergoing hysterectomy in Liverpool Women's Hospital and Lancashire Teaching Hospitals Trusts from 2009-2014. The histological type and grade of EC specimens were assigned by experienced gynaecological pathologists according to the International Federation of Gynaecology and Obstetrics (FIGO) (Zaino et al., 1995). ECs were categorised as low grade (LGEC); including Grade 1 and grade 2 endometrioid EC or high grade (HGEC); including grade 3 endometrioid, serous, clear cell carcinoma and carcinosarcoma (Voss et al., 2012) for subsequent analysis of IHC data (Table 3.1). Proliferative phase specimens were assigned according to last menstrual date (LMP) and histological criteria (Noyes et al., 1950; Dallenbach-Hellweg et al., 2010). All samples were split into 2; one was fixed for 24 h in 4% (v/v) buffered formalin then paraffin embedded for immunohistochemical staining and the other part was immediately placed into RNAlater® (Sigma, Dorset, UK) for RNA extraction for PCR.

Patient clinicopathological and demographic details were retrieved by review of hospital notes and clinical databases. None of the patients received hormonal treatments, chemotherapy or pelvic radiation prior to surgery.

Table 3.1 Demographic features of study groups.

Study groups	Number of cases	%	Age* (years)	BMI** kg/m ²
Proliferative phase	14		39 (30-47)	26.7 (17.5-45.5)
Postmenopausal	14		68.5(57-79)	26.3 (22.7-35.8)
Endometrial hyperplasia	12		50 (37-67)	29.7 (34.3)
<i>Without cytological atypia</i>	4		55 (50-62)	25.75 (23.6-53.2)
<i>With cytological atypia</i>	8		52 (37-67)	34 (27.9-57.8)
Endometrial cancer	85		67 (41-89)	30 (20.2-54.4)
LGEC	37	43.5	63.5 (41-84)	30.8 (21.6-46.1)
<i>Grade1 endometrioid</i>	19	22.4	63 (46-84)	35.7 (21.6-46.1)
<i>Grade2 endometrioid</i>	18	21.2	63.5 (41-83)	29 (22.3-54.4)
HGEC	48	56.5	70 (51-89)	29.6 (20.2-54.4)
<i>Grade3 endometrioid</i>	15	17.6	69 (51-83)	26.7 (22.1-42.7)
<i>Serous</i>	7	8.2	68 (64-82)	29.5 (24.8-34.9)
<i>Clear cell</i>	12	14.1	69 (52-82)	29.9 (25.4-31.5)
<i>Carcinosarcoma</i>	14	16.5	54.5 (59-89)	28.6 (20.2-37.2)
Metastatic lesions	16		68 (41-89)	**

* Data expressed as median (range), ** BMI data were available for only 60 cases.

3.2.2 Immunohistochemistry (IHC)

After antigen retrieval at pH6 as previously described in chapter 2.7.1.1, 3 µm formalin fixed paraffin embedded tissue sections were immunostained with anti-human steroid receptor antibodies and Ki67; antibody sources, concentrations and incubation conditions are detailed in (Table 3.2). Detection was with the ImmPRESS polymer based system and visualisation was with ImmPACT DAB (Vector Laboratories, Peterborough, UK) used in accordance with the manufacturer's instructions. Sections were lightly counterstained in Gill 2 Haematoxylin (Thermo Scientific, Runcorn, UK), dehydrated, cleared and mounted in synthetic resin. Matching isotype (0.5 µg/mL) replaced the primary

antibody as a negative control, with internal positive controls performed in each staining run.

Table 3.2 Primary antibodies and their immunohistochemistry conditions.

Primary Ab	Type	Clone	Supplier	HIAR* (min)	Dilution	Incubation conditions	
						Time (hour)	Temp (°C)
AR	Monoclonal	441	DAKO ¹	2	1:50	20	4
PR	Monoclonal	PgR 636	DAKO	2	1:1000	0.5	18
ER α	Polyclonal		Abcam ²	2	1:50	2	18
ER β	Monoclonal	PPG5/10	Serotec ³	2	1:50	20	4
Ki67	Monoclonal	MM1	Leica ⁴	4	1:200	20	4

*Heat induced antigen retrieval by pressure cooking in citrate buffer pH 6 (Hapangama et al, 2012). ¹ Ely, Cambridgeshire, UK ; ² Cambridge, UK ; ³ Oxford, UK ; ⁴ Newcastle upon Tyne, UK .

3.2.3 Analysis of IHC staining

Immunostaining for the four steroid receptors was assessed semi-quantitatively using a 4-tiered Liverpool endometrial steroid quick score (LESQS). Initially, we recorded the percentage of positively stained target cells in each of four intensity categories. H-score was calculated using the formula described by McCarty (1986):

$$Hscore = \sum pi(i+1)$$

where i represents the intensity of staining with a value of 0, 1, 2 or 3 (no staining, weak, moderate, strong staining) and P_i ; varies from 0 to 100%. We then proposed a 4 tiered Liverpool endometrial steroid quick score (LESQS) based on the homogenous distribution of the observations in the arbitrary cut-off categories within our endometrial samples (1%-10% =1, 11%-20% =2, 21%-40% =3, and >40%=4). The score for the proportion of positive cells was then multiplied by the staining intensity categories and added to give a final score out of 12. For example, if 40% of the cells were weakly positive (3x1=3), 20% moderately positive (2x2=4),

10% strongly positive ($1 \times 3 = 3$) and the remaining 30% were negative ($3 \times 0 = 0$), the total score will be: $3 + 4 + 3 + 0 = 10$

We also employed two well established quick scores, the Allred score (Allred et al., 1998) and the immunoreactive score of Remmele and Stegner (IRS) (Remmele and Stegner, 1987) to identify the semi-quantitative scoring system that best reflects the steroid receptor profile in the endometrium. The Allred score is a five-tier score rated as ($\leq 1\% = 1$, $1-10\% = 2$, $11-33\% = 3$, $34-66\% = 4$ and $67-100\% = 5$); the total score out of 8 is obtained by adding the score of the proportion with the predominant staining to the score of the intensity 0, 1, 2 or 3 (no staining, weak, moderate, strong staining). Therefore, for the example above, 40% (proportion score 4) of the sample expressed weak staining (intensity score 1) which is the predominant staining intensity; thus, the total score is $4 + 1 = 5$

The IRS score is a four-class score where 0 = no staining; 1 = $\leq 10\%$ staining; 2 = $11-50\%$ staining; 3 = $51-80\%$ staining; and 4 = $\geq 81\%$ staining. The total score out of 12 is obtained by multiplying the intensity score 0, 1, 2 or 3 by the proportion score. For the same example, $(2 \times 1) + (2 \times 2) + (1 \times 3) + (2 \times 0) = 9$

The Ki67 proliferative index (PI) was evaluated as the percentage of immunopositive cells, of any intensity. The entire section was evaluated at 400X magnification as previously described (Al Kushi et al., 2002).

Epithelial and stromal cell staining was scored separately in PM and malignant endometrium and stratum basalis of healthy PP endometrium by two independent observers (AMK and DKH). Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

For the purpose of description, scores 1-4 were considered as low, 5-8 as moderate, and 9-12 as high levels of expression. For survival analysis and correlation with clinicopathological analysis, data were subclassified as immunopositive when $>10\%$ of the neoplastic cells expressed the target protein at any intensity (LESQS ≥ 2) and immunonegative if < 2 (Pertschuk et al., 1996).

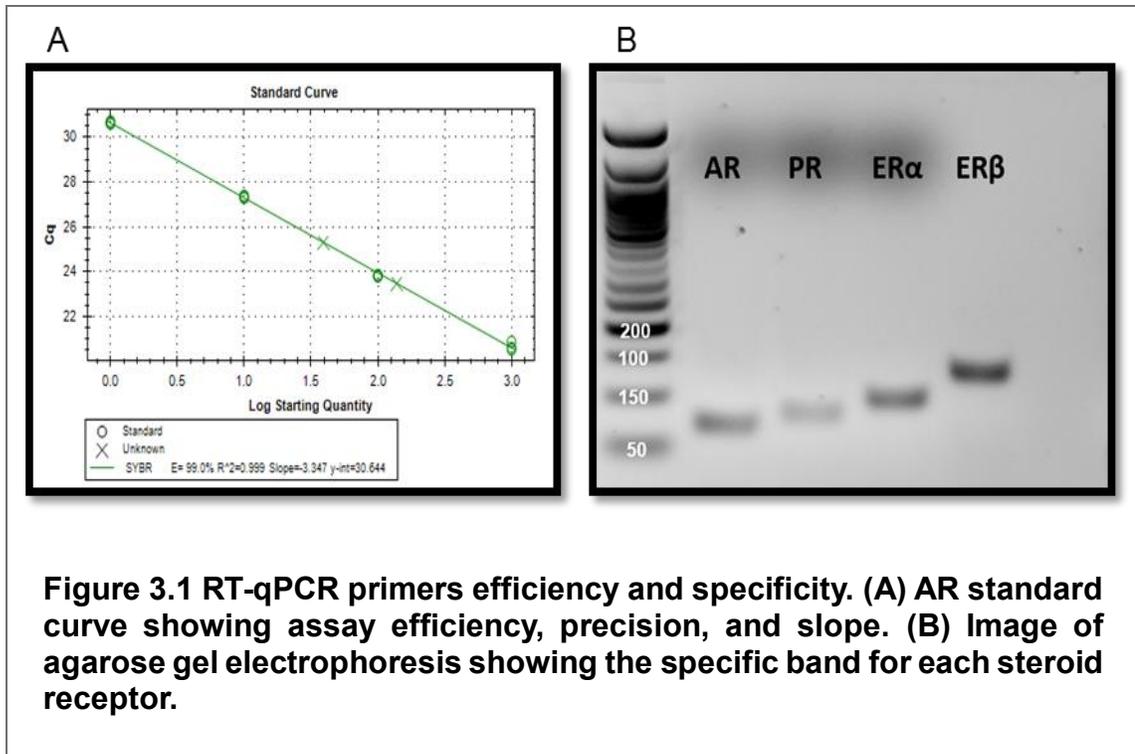
3.2.4 RT-qPCR

Total RNA from tissue samples was extracted using TRIzol® Plus RNA Purification System (Life Technologies, Paisley, UK), and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Total RNA was reverse transcribed using AMV First Strand cDNA synthesis kit (New England Bio Labs, Hertfordshire, UK) after DNase treatment (DNase I (#M0303), New England Bio Labs, Hertfordshire, UK), using the manufacturer's protocol. cDNA was amplified by qPCR using JumpStart SYBR Green supermix (Sigma, Dorset, UK) and the Light Cycler 96 Roche Real-Time System (Roche Diagnostics Ltd. Burgess Hill, UK). Primers are listed in Table 3.3.

Table 3.3 Primer sequences used for RT- qPCR amplification.

Primer	Sequence	Amplicon	Efficiency	Reference
AR	F: 5'AGGATGCTCTACTTCGCCCC3' R: 5'CTGGCTGTACATCCGGGAC3'	72	99%	Pichler <i>et al.</i> , 2013
PR	F: 5'CAGTGGGCGTTCCAAATGA3' R: 5'TGGTGAATCAACTGTATGTCTTGA3'	83	101.3%	Henderson <i>et al.</i> , 2003
ER α	F: 5'TGATTGGTCTCGTCTGGCG3' R: 5'CATGCCCTCTACACATTTTCCC3'	101	92.5%	Henderson <i>et al.</i> , 2003
ER β	F: 5'CCTGGCTAACCTCCTGATGCT3' R: 5'CCACATTTTGCACATTCATGTTG3'	92	90.3%	Henderson <i>et al.</i> , 2003
YWHAZ	F: 5'CGTACTTGGCTGAGGTTGCC3' R: 5'GTATGCTTGTGTGACTGATCGAC3'	69	91.1%	Marullo <i>et al.</i> , 2010

For each target and reference, a standard curve was generated and efficiency was determined (Figure 3.1A). Products were resolved after the first amplification on agarose gel (Figure 3.1B) to confirm the specificity of the primers and rule out any off targets. Relative transcript expression was calculated by the $\Delta\Delta CT$ method, normalised to the reference gene YWHAZ (Sadek *et al.*, 2011) using Biogazelle qbase+ software (Biogazelle NV, Zwijnaarde, Belgium).



3.2.5 Statistical analysis

Statistical differences between groups were calculated by non-parametric tests (Kruskal–Wallis, and/or Mann–Whitney U-test or Wilcoxon signed rank test) using Statistical Package for the Social Sciences (SPSS) version 21. Descriptive values were presented as median and range. The correlations between immun-expression scores were examined with Spearman test and association between immuno-scores and the multiple clinicopathological parameters with Pearson Chi-square. Disease free (DFS), cancer specific (CSS) and overall (OS) survivals were calculated from the date of surgery to the date of recurrence/ death or the date on which the patient was last seen as described in chapter 2.9. For survival analysis, each parameter was categorised and survival curves were obtained using the Kaplan-Meier method. The Cox proportional hazards regression model was used to identify the independent prognostic factors. Only variables with $P <$

0.05 in the univariate analysis were included in the multivariate model. $P < 0.05$ was considered significant.

3.3 Results

3.3.1 Demographic data

Patient demographics are detailed in Table 3.1. Women with HGEC were significantly older than those with LGEC ($P < 0.0001$). Patients with EHA were significantly younger than those with LGEC ($P = 0.016$). Healthy PM controls had the lowest BMI compared with women who had EHA ($P = 0.007$) and LGEC ($P = 0.022$). There was no significant difference in BMI between LGEC and HGEC.

3.3.2 LESQS

The LESQS, Allred score and IRS were correlated with the H score of corresponding samples in a subset of 37 EC samples which showed the lowest, intermediate and highest steroid expression levels. Compared with the H score, the LESQS showed the highest correlation for AR and ER α and good correlation for PR and ER β Table 3.4. The LESQS was therefore preferentially chosen for analysis of expression of the four steroid hormone receptors in our samples.

Table 3.4 Correlation between H score of AR, PR, ER α and ER β and LESQS, IRS and Allred scores in epithelial and stromal compartments.

H score	Epithelial expression			Stromal expression		
	LESQS	IRS	Allred	LESQsS	IRS 3	Allred
AR	0.968	0.948	0.96	0.962	0.951	0.935
PR	0.93	0.976	0.935	0.967	0.946	0.951
ER α	0.97	0.865	0.907	0.913	0.823	0.713
ER β	0.746	0.715	0.747	0.852	0.761	0.943

Abbreviations: Liverpool endometrial steroid quick score (LESQS), immunoreactive score (IRS).

3.3.3 IHC analysis of AR and other steroid receptor expression

All 4 steroid receptors were expressed by the endometrium (Figure 3.2). The main focus of interest, AR, was expressed by both the epithelium and stroma. At a subcellular level, both cytoplasmic and nuclear AR staining were observed; only nuclear immunostaining suggesting transcriptionally active AR with functional relevance was scored and semi-quantified.

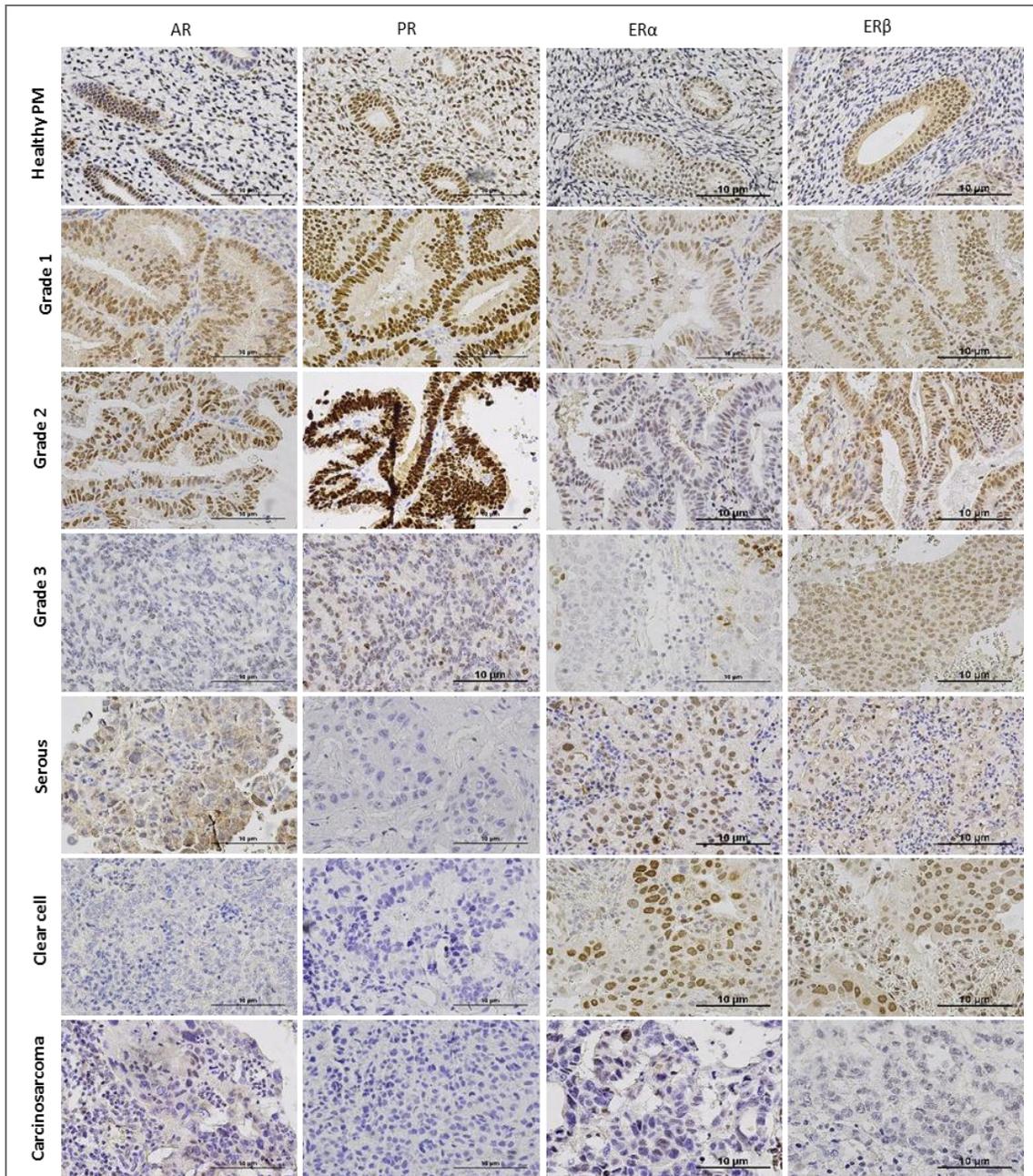


Figure 3.2 Representative photomicrographs of immunolocalisation of AR, PR, ER α and ER β in human endometrium. Healthy postmenopausal (PM) endometrium, grade 1-3 endometrioid carcinoma, serous carcinoma, clear cell clear cell carcinoma and carcinosarcoma were immunostained for AR, PR, ER α and ER β . Positive staining appears brown. Magnification 400X. Scale bar 10 μ m

3.3.3.1 Healthy postmenopausal endometrial epithelial cells acquire AR and preserve ERs and PR

The dominant steroid receptor in both epithelium and stroma of the healthy endometrium was PR. In PP endometrium, AR expression was largely limited to stromal cells in both the stratum basalis and functionalis with absent epithelial AR staining. In contrast, the most striking feature of non-proliferating PM endometrium was the emergence of nuclear AR ($P < 0.001$) in the epithelial cells (Figure 3.3A). Compared with epithelial cells in the stratum basalis of PP, PM epithelial cells also expressed significantly higher levels of ER α ($P = 0.035$) (Figure 3.3C and E); however, there were no differences in ER β and PR expression scores or in the ER α /ER β ratio. The stromal expression scores for both PM and stratum basalis of PP were similar for all the steroid receptors examined (Figure 3.3E).

3.3.3.2 AR expression and ER α /ER β ratio are increased in atypical endometrial hyperplasia

PR immunoexpression was the strongest of the steroid receptors in both epithelial and stromal compartments of EH (Figure 3.3E). Interestingly, compared with the epithelium of PP, nuclear AR expression was significantly higher in the epithelial cells of EHA ($P = 0.025$) but no significant change was observed when compared to PM epithelium (Figure 3.3A and C). Endometrial stroma of EHA showed significant loss of AR expression compared with the stroma of PP ($P < 0.0001$). The general trend of ER α expression in the epithelium of EHA was higher than that of PP ($P = 0.076$) and PM ($P = 0.547$). In contrast, the trend of ER β expression in EHA was lower than that of PP ($P = 0.128$) and significantly lower compared with PM endometrium ($P = 0.014$, Figure 3.3C). Thus, ER α /ER β in EHA was generally higher than that of the normal endometrium; this was significant compared to PP ($P = 0.041$).

In common with AR, the expression of both ER α ($P = 0.043$) and ER β ($P = 0.045$) in the stroma of EHA compared with PM endometrium were significantly decreased

whereas the expression of PR was increased ($P=0.02$). The immunoscores of PR were preserved in the epithelial cells of EHA at a level that was comparable with that of healthy PP and PM samples. The proliferative index, assessed by Ki67 immunopositivity, of epithelial cells in EHNA and EHA was similar to that of PP epithelium but, as expected, was significantly higher than PM epithelium (EHNA $P=0.016$, EHA $P=0.017$).

3.3.3.3 AR, PR and ER β are downregulated in the high grade endometrial cancer

We chose healthy PM tissue as the healthy comparator for steroid receptor expression scores of EC samples. ER β was the predominant steroid receptor expressed in both LGEC and HGEC (Figure 3.2 and 3.34C). AR ($P=0.10$, Figure 3.2, 3.3A and C) and ER α ($P=0.05$, Figure 3.3C) staining scores showed a trend to be increased in LGEC with a simultaneous reduction in PR ($P=0.08$, Figure 3.3C). This was associated with a significant reduction in stromal expression of AR ($P<0.0001$, Figure 3.3B), ER α ($P<0.0001$) and PR ($P<0.0001$) when compared with healthy PM controls. There was no significant change in the scores of ER β (stromal and epithelial) in LGECs compared with healthy controls, although the ER α /ER β ratio was higher in LGEC (PP, $P<0.0001$; PM, $P=0.02$).

The epithelial cells of HGEC and the surrounding stroma showed a general reduction in expression of all four steroid receptors compared with healthy PM tissue (Figure 3.3E). Compared with LGEC, in HGEC the scores of epithelial AR ($P<0.0001$), PR (<0.0001) and ER β ($P=0.035$) were significantly lower (Figure 3.2, 3.3A and C). Within the subtypes of HGEC, the most pronounced loss of AR was observed in the clear cell carcinoma group ($P=0.001$); albeit the expression of ER α scores in the same group remained moderate to strong (Figure 3.3D). Weak to moderate cytoplasmic AR was observed in 33% (11/33) HGEC in the absence of nuclear staining (Figure 3.1, serous).

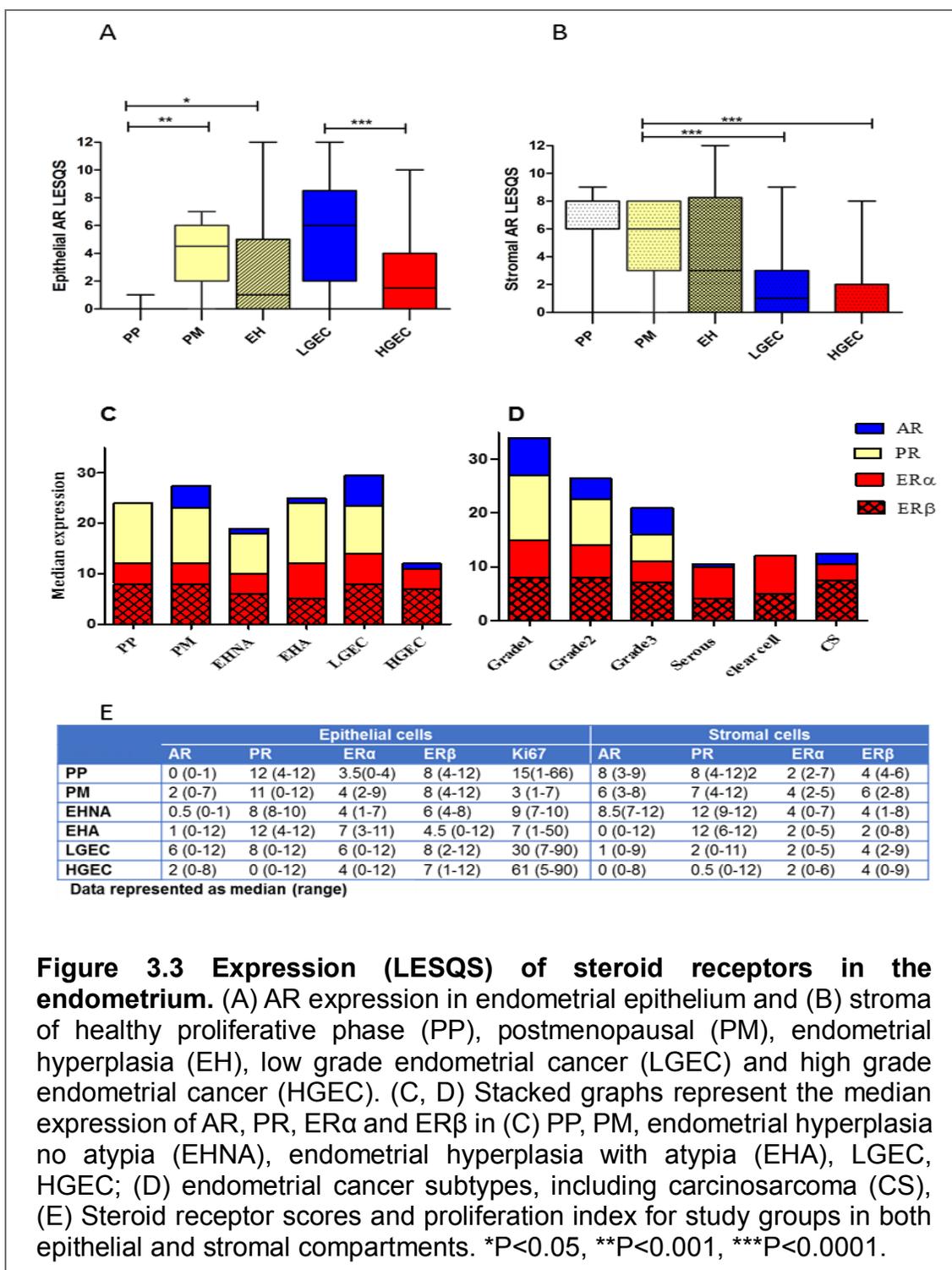
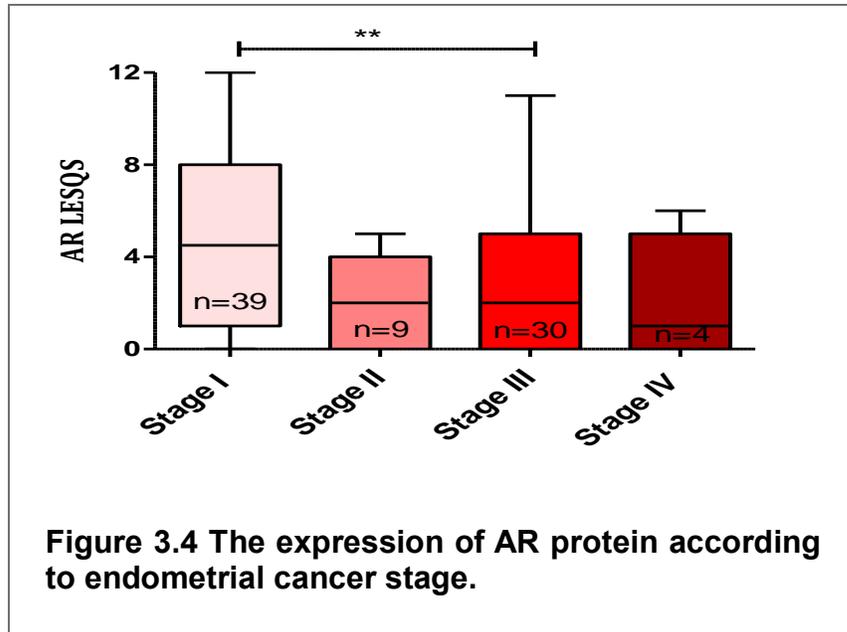


Figure 3.3 Expression (LESQS) of steroid receptors in the endometrium. (A) AR expression in endometrial epithelium and (B) stroma of healthy proliferative phase (PP), postmenopausal (PM), endometrial hyperplasia (EH), low grade endometrial cancer (LGEC) and high grade endometrial cancer (HGEC). (C, D) Stacked graphs represent the median expression of AR, PR, ER α and ER β in (C) PP, PM, endometrial hyperplasia no atypia (EHNA), endometrial hyperplasia with atypia (EHA), LGEC, HGEC; (D) endometrial cancer subtypes, including carcinosarcoma (CS), (E) Steroid receptor scores and proliferation index for study groups in both epithelial and stromal compartments. *P<0.05, **P<0.001, ***P<0.0001.

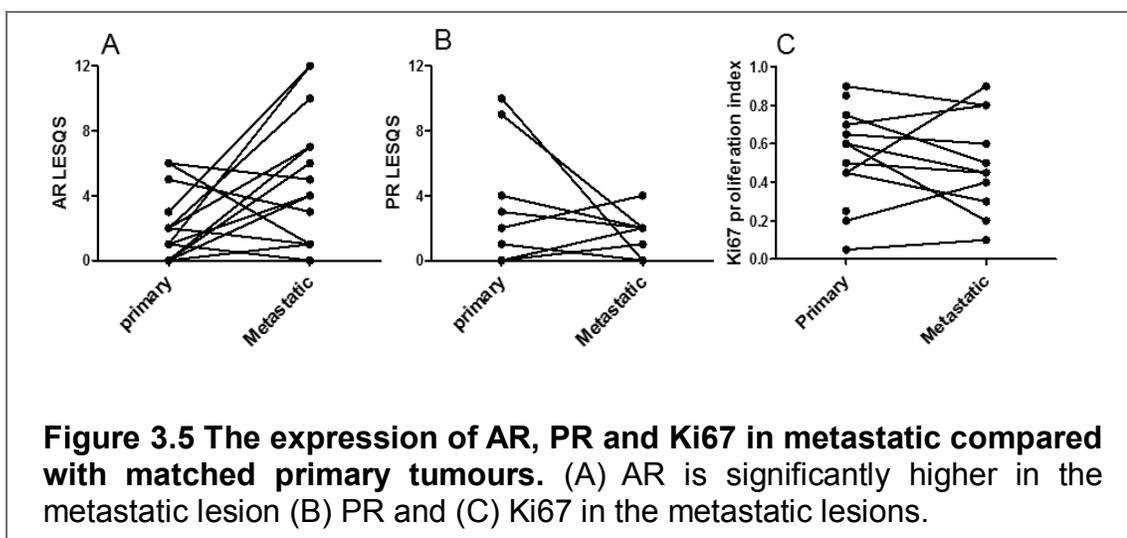
The expression scores of both PR and ER β in HGEC epithelial cells (PR P<0.0001, ER β P=0.003) and surrounding stroma (PR P<0.0001, ER β P=0.02) (Figure 3.3C) were significantly lower than healthy PM counterparts. Interestingly,

in non-endometrioid HGEC, 26/33 (78.8%) showed loss of PR whilst all were ER β + and 30/33 (90.9%) were also ER α +. PR loss was limited to only 5/15 (33.3%) of the endometrioid HGEC. Expression of AR in HGEC was comparable with PR; 16/33 (48.5%) were AR negative and 13 of these were also negative for PR. Furthermore, only AR expression (not PR) reduced significantly with advanced FIGO stage (stage I vs. stage III, P=0.006, Figure 3.4).



3.3.3.4 Metastatic lesions acquire AR

Nuclear AR was observed in 10/16 (62.5%) (Figure 3.5A) metastatic lesions and expression scores were significantly higher compared with their matched primary lesions (P=0.03). By contrast, only 6/15 (40%) (Figure 3.5B) of metastatic lesions expressed PR. Although the median expression of Ki67 was lower in metastatic lesions (45%) than in the matched primary tumour (60%), the difference in the expression pattern of both PR and Ki67 between the two groups was not statistically significant (Figure 3.5C).



3.3.3.5 AR expression positively associates with favourable prognostic factors

In EC, the immunoscores of AR correlated positively with PR ($r=0.63$, $P<0.0001$), whilst there was a negative correlation with Ki67 ($r=-0.43$, $P=0.0004$, Table 3.5). When expression of steroid receptors (as positive or negative) was correlated with each clinicopathological parameter, both AR and PR expression correlated positively with well-differentiated tumours and those without cervical invasion, yet only AR immunoscores showed a positive correlation with early FIGO ($P=0.048$, Table 3.6).

Table 3.5 Correlation of AR, PR, ER α , ER β and Ki67 in endometrial cancer samples.

		AR	PR	ER α	ER β	Ki67
AR	r		0.63	0.23	0.38	-0.43
	P		<0.0001	0.0342	0.0003	0.0004
PR	r	0.63		0.23	0.33	-0.40
	P	<0.0001		0.035	0.002	0.001
ERα	r	0.2	0.2		0.23	-0.19
	P	0.034	0.035		0.038	0.128
ERβ	r	0.38	0.33	0.23		-0.04
	P	0.000	0.002	0.038		0.775

Intriguingly, concurrent loss of AR and PR showed a significant positive correlation with higher tumour grades ($P<0.0001$), late FIGO stages ($P=0.004$), deep

myometrial invasion ($P=0.003$), extrauterine invasion ($P=0.048$) and cervical invasion ($P<0.0001$). ER α did not show a significant correlation with clinicopathological parameters; however, a low ER α /ER β ratio was associated with invasion of the cervical stroma ($P=0.041$) and showed a trend to be associated with advanced stage tumours ($P= 0.057$).

Table 3.6 Contingency analysis of steroid receptor expression in relation to known prognostic factors.

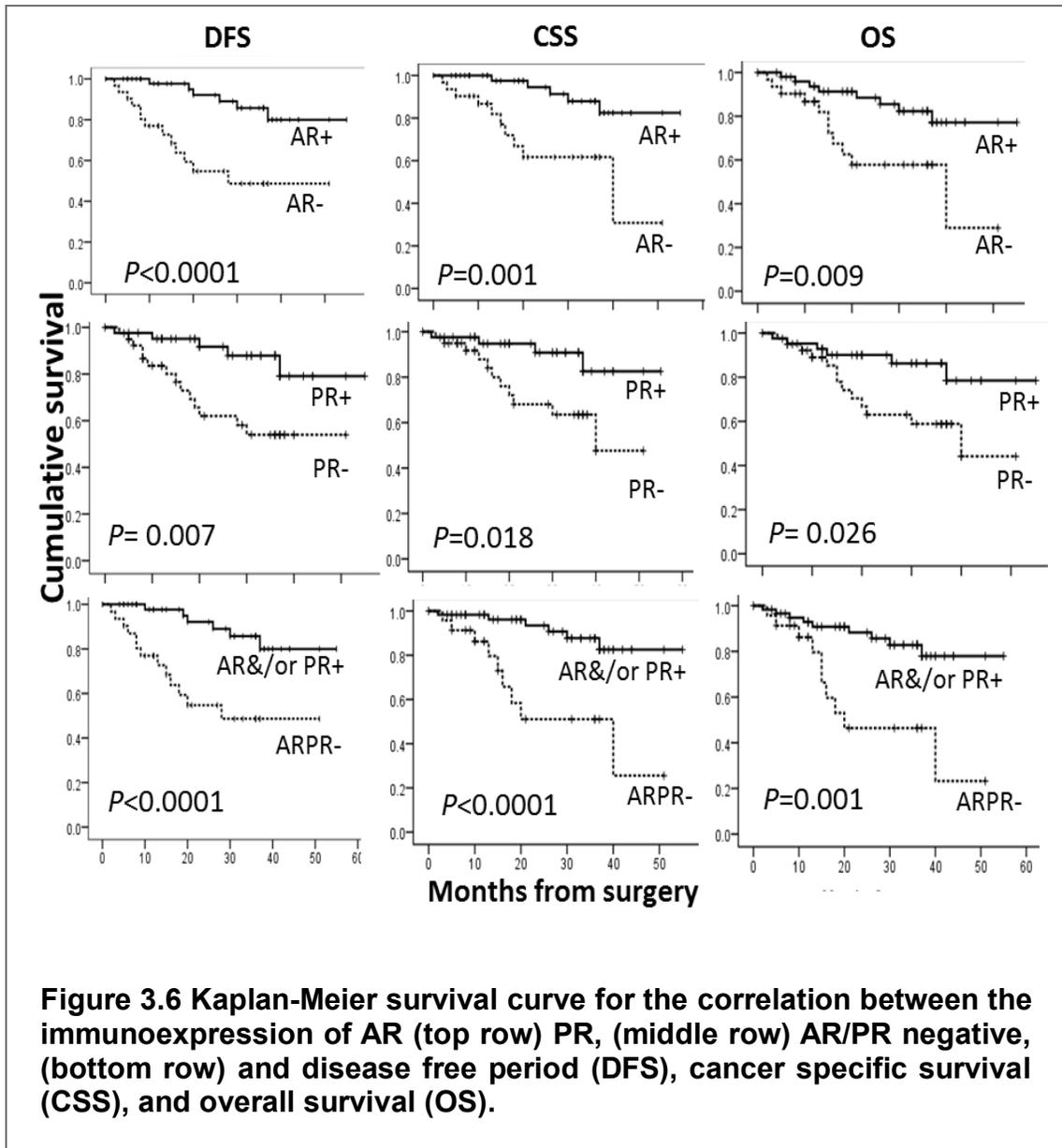
Receptor status	Number (n=)	Age		BMI*		FIGO stage		Grade		LVI		Myometrial invasion		Extra-uterine invasion		Lymph node status*		Cervical stromal invasion	
		≤65	>65	≤30	>30	I-II	II-IV	LG	HG	-	+	≤50%	>50%	No	Yes	No	Yes	No	Yes
		AR	-	32	11 21	12 9	13 18	9 23	15 13	15 15	16 15	4 7	15 15	-	-	-	-	-	-
	+	54	26 28	18 21	34 19	29 25	28 23	31 23	37 15	5 4	45 9	-	-	-	-	-	-	-	-
	P		0.263	0.417	0.048	0.021	0.91	0.513	0.073	0.653	0.001	-	-	-	-	-	-	-	-
PR	-	43	15 28	16 13	21 22	9 34	22 19	19 23	24 17	7 6	24 18	-	-	-	-	-	-	-	-
	+	43	22 21	14 17	26 15	29 14	21 17	27 15	29 13	2 5	36 6	-	-	-	-	-	-	-	-
	P		0.127	0.438	0.179	<0.0001	0.886	0.079	0.319	0.374	0.004	-	-	-	-	-	-	-	-
AR-PR-	no	55	26 29	17 24	36 17	33 22	29 21	36 18	38 15	4 6	47 7	-	-	-	-	-	-	-	-
	yes	31	11 20	13 6	11 20	5 26	14 15	10 20	15 15	5 5	13 17	-	-	-	-	-	-	-	-
	P		0.28	0.052	0.004	<0.0001	0.403	0.003	0.048	1	<0.0001	-	-	-	-	-	-	-	-
ER α	-	11	4 7	3 5	5 6	2 9	3 7	6 5	5 6	2 3	7 4	-	-	-	-	-	-	-	-
	+	75	33 42	27 28	42 31	36 39	40 29	40 33	48 24	7 8	53 20	-	-	-	-	-	-	-	-
	P		0.633	1	0.524	0.102	0.172	0.988	0.173	1	0.721	-	-	-	-	-	-	-	-
ER β	-	1	0 1	5 8	0 1	0 1	1 0	1 0	0 1	1 0	1 0	-	-	-	-	-	-	-	-
	+	85	37 48	25 22	47 36	38 47	42 36	45 38	53 29	8 11	59 24	-	-	-	-	-	-	-	-
	P		1	0.35	0.44	0.558	1	1	0.361	0.45	0.714	-	-	-	-	-	-	-	-
ER α /ER β	≤1	23	9 14	8 6	8 13	7 16	11 11	11 11	11 10	3 4	12 10	-	-	-	-	-	-	-	-
	>1	63	28 35	22 24	39 24	31 32	24 37	35 26	42 20	5 8	48 14	-	-	-	-	-	-	-	-
	P		0.66	0.54	0.057	0.121	0.482	0.551	0.205	1	0.041	-	-	-	-	-	-	-	-

*BMI data are limited to 60 cases and lymph node status was reported in 20 cases only.

3.3.3.6 Loss of AR adversely influences patient outcome

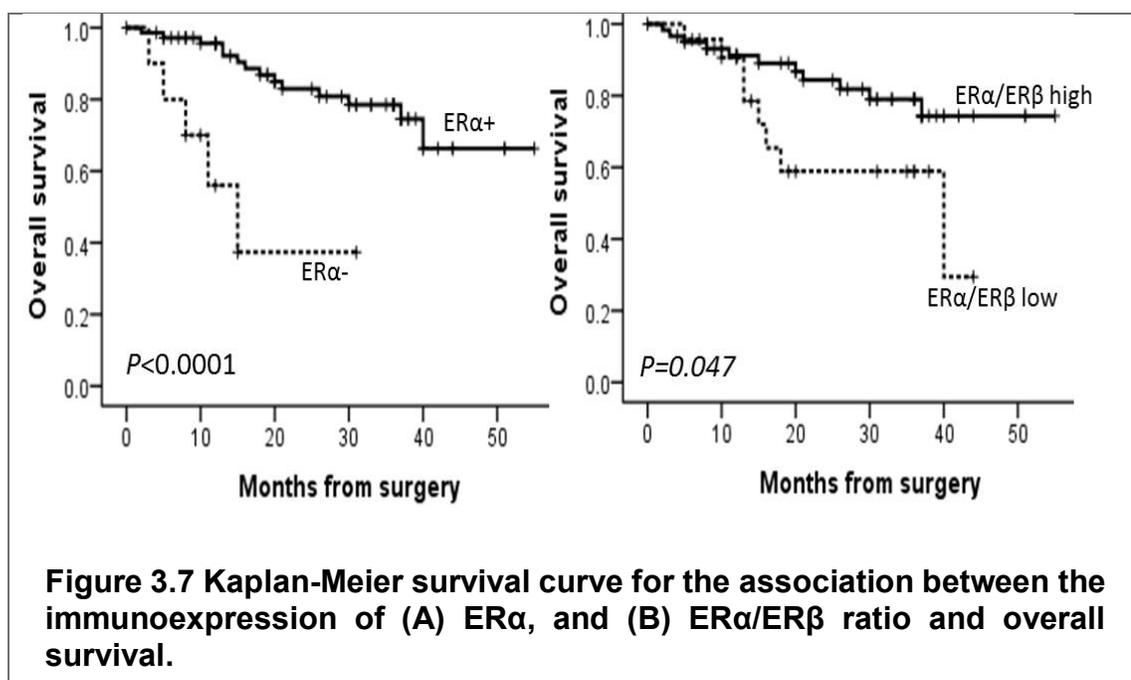
Follow up data were available for all EC patients. By June 2016 the median follow-up was 24 months, ranging between 6-55 months. During the follow-up period,

there were 5 recurrent tumours and 19 deaths (15 as a result of disease progression and 4 from other causes).



As Figure 3.6 shows, a significant reduction in patient survival was identified in the AR negative group (DFS, $P < 0.0001$; CSS, $P = 0.001$; and OS, $P = 0.009$) and PR negative group (DFS, $P = 0.007$; CSS, $P = 0.018$; and OS, $P = 0.026$). A subset of EC which were negative for both AR and PR showed a further decline in DFS ($P < 0.0001$), CSS ($P < 0.0001$) and OS ($P = 0.001$). Moreover, cancers with ER α -

($P < 0.0001$) and low ER α /ER β ratio ($P = 0.047$) associated with shorter OS than those with a ER α + and high ER α /ER β ratio. ER β expression did not show a significant association with clinical outcome (Figure 3.7). Univariate analysis has shown that HGEC, deep myometrial invasion, LVSI, cervical stromal invasion, extra-uterine invasion, loss of AR, loss of PR and combined AR/PR loss were significantly associated with cancer progression, cancer specific and overall death. Advanced stage III and IV was associated cancer progression and overall death, older age and low ER α /ER β was associated with cancer progression and loss of ER α was associated with overall death. BMI and ER β did not show association with patient outcome (Table 3.7).



Furthermore, Cox regression model confirmed that only AR (DFS, $P = 0.013$), ER α (OS, $P = 0.002$), tumour grade (OS, $P = 0.024$) and cervical stromal invasion (DFS, $P = 0.001$; CSS, $P < 0.0001$, OS, $P = 0.024$) were independent prognostic indicators of patient outcome; (Table 3.7).

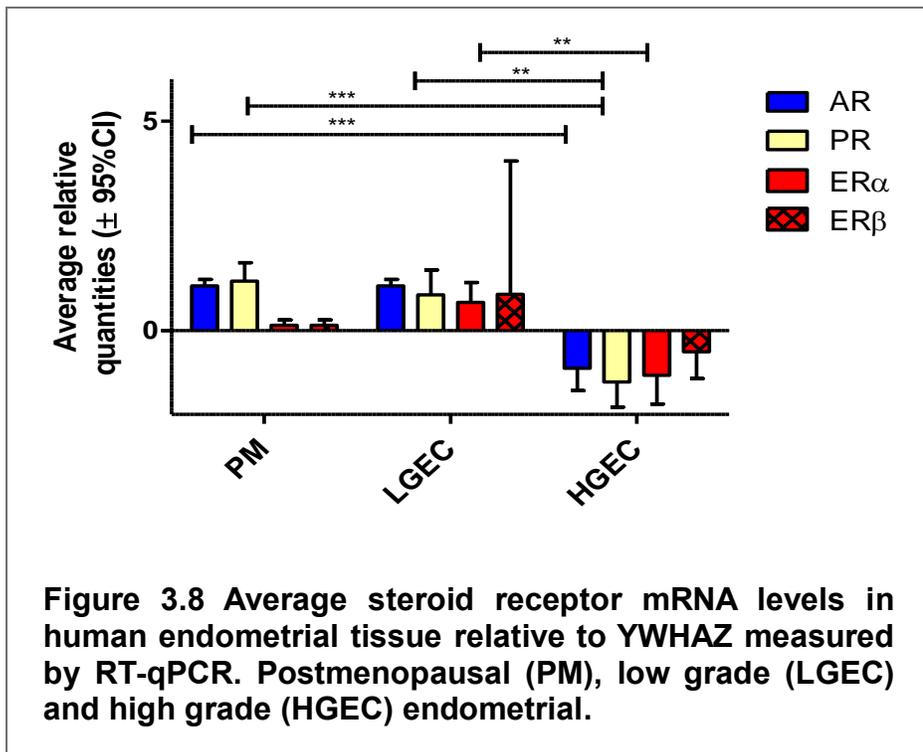
Table 3.7 Univariate and multivariate analyses of disease free, cancer specific and overall survivals for endometrial cancer patients.

Variables	Total number	Disease free survival			Cancer specific survival			Overall survival		
		Univariate		Multivariate	Univariate		Multivariate	Univariate		Multivariate
		P	P	HR* (95% CI)	P	P	HR* (95% CI)	P	P	HR* (95% CI)
Age (years): ≤65 vs >65	97	0.047	0.724	-	0.101	removed	-	0.136	removed	-
BMI: ≤30 vs >30	65	0.553	removed	-	0.766	removed	-	0.572	removed	-
Tumour stage: I-II vs III-IV	97	0.023	0.142	-	0.064	removed	-	0.013	0.985	-
Tumour grade: LG vs HG	97	0.003	0.386	-	0.024	0.105	-	0.017	0.024	6 (1.26 -28.27)
Lympho-vascular invasion: - vs +	97	0.013	0.203	-	0.029	0.158	-	0.008	0.120	-
Myometrial invasion : ≤50% vs >50%	93	0.003	0.05	3 (1-9)	0.012	0.099	-	0.012	0.375	-
Cervical stromal invasion: - vs +	97	0.000	0.001	5.7(2.-16.8)	0.001	0.0001	10 (3.1-33.4)	0.002	0.024	3.23 (1.2-9.1)
Extra uterine invasion: - vs +	96	0.004	0.912	-	0.016	0.335	-	0.009	0.585	-
AR expression: - vs +	86	0.001	0.013	0.4 (0.1-0.9)	0.004	0.082	-	0.014	0.793	-
PR expression: - vs +	86	0.011	0.732	-	0.027	0.302	-	0.034	0.863	-
AR-PR- expression: - vs +	86	0.000	0.861	-	0.001	0.146	-	0.003	.957	-
ERα expression: - vs +	86	0.897	removed	-	0.755	removed	-	0.001	0.002	0.2 (0.05 - 0.5)
ERβ expression: - vs +	86	0.897	removed	-	0.879	removed	-	0.881	removed	-
ERα/β ratio: ≤1 vs >1	86	0.021	0.098	-	0.007	0.107	-	0.057	removed	-

*Hazard ratio

3.3.4 Steroid receptor mRNA levels reflect their protein expression

Consistent with the IHC results, a general decline in mRNA levels for all steroid receptors was observed in HGEC. This was significant for AR ($P=0.0002$) when compared with PM endometrium, and $ER\alpha$ ($P=0.003$) when compared with LGEC. PR transcript level in HGEC was significantly lower than both PM ($P=0.0002$) and LGEC ($P=0.001$). The change in $ER\beta$ mRNA was not significant (Figure 3.8). Furthermore, AR ($r=0.59$, $P=0.015$) and PR ($r=0.74$, $P=0.001$) mRNA levels also showed a strong correlation with their protein expression scores.



3.4 Discussion

To our knowledge, this is the first comprehensive report comparing AR expression in healthy PM endometrium with healthy premenopausal PP endometrium and all EC subtypes including non-endometrioid type II (serous, clear cell, and carcinosarcoma) ECs and metastatic lesions. We have also described the

contemporaneous expression scores for ER α , ER β , and PR in serial sections of the same endometrial samples, allowing inferences to be made regarding their functional interplay in both healthy endometrium and in endometrial carcinogenesis.

Previous reports on the expression of steroid receptor proteins in normal and pathological endometrium have used different semi-quantification methods (Mertens et al., 2001; Critchley et al., 2002; Mylonas et al., 2007; Zannoni et al., 2013). Of the available quickscores, Allred and IRS have been commonly used to analyse steroid receptors in the endometrium (Mylonas et al., 2007; Zannoni et al., 2013) but these two systems were optimised for ER and PR expression (not AR) specifically in breast tissue. We propose an LESQS, which is optimised for both normal and neoplastic endometrium. Importantly, LESQS had the best correlation with a standard *H*score for AR and ER α compared with both Allred and IRS scores for either epithelial or stromal compartments. LESQS also showed high correlation with the *H*score for PR and ER β , similar to the Allred score correlation. In order to gain better insight into hormone actions, all cognate steroid receptors (AR, ER α , ER β and PR) need to be assessed using the same scoring system; hence, the optimised LESQS was chosen.

PM endometrium is composed of inactive glands lying in a compact stroma that morphologically resembles the stratum basalis of the premenopausal endometrium (McCluggage, 2011). Moreover, the endometrium after menopause is characterised by complete loss of the stratum functionalis; therefore, we compared the expression of steroid receptors in PM endometrium with that of PP stratum basalis. Interestingly, compared with the stratum basalis of PP, we found significantly higher AR and ER α immunoexpression in the epithelium of PM endometrium but similar ER β and PR immunoexpression. Horie et al. (1992) reported AR expression (albeit in a sample size of $n=4$) in the epithelium of PM endometrial, which is consistent with our results, but they indicated that this was similar to the expression of AR in stratum basalis of PP. Observational bias cannot be excluded in such a small sample size particularly in the absence of

quantification which may explain the differences in conclusion between the two studies. In contrast, a previous report comparing PM endometrium with PP stratum functionalis suggested a decrease of ER α , ER β and PR expression in glandular epithelial cells of PM endometrium (Mylonas et al., 2007). The hormonal milieu of PM women is characterised by the absence of progesterone and oestradiol, the presence of low levels of circulating oestrone (Sivridis and Giatromanolaki, 2004) and persisting levels of androgens from the adrenals, which may support the maintenance of endometrial PR and ER expression. Although there are reports suggesting focal and weak AR expression appearing in the glandular epithelium of late secretory phase, presumed to be associated with progesterone withdrawal (Horie et al., 1992; Mertens et al., 2001); the most prominent feature of premenopausal secretory phase endometrium is the high immunoexpression of AR in the stroma. This is in contrast with PM endometrium, where dominant AR staining was observed in the epithelium. Furthermore, administration of mifepristone, an anti-progesterone, has been associated with upregulation of AR immunoexpression in both endometrial stromal and epithelial cells of premenopausal primate (Brenner and Slayden, 2004), yet we observed low AR immunoexpression in the stromal AR of PM compared with that of PP. Since by definition, PM endometrium has not been exposed to progesterone for at least 12 months, the appearance of AR in the glandular epithelium of PM endometrium cannot be equated merely to progesterone withdrawal. To complicate matters further, mifepristone is not only a progesterone antagonist but also has anti-androgenic and glucocorticoid properties (Hapangama, 2003). Therefore, the appearance of AR in PM epithelium may be induced by the action of either oestrone via ER α or androgens via AR (Fujimoto et al., 1994; Lovely et al., 2000). Interestingly, the upregulation of AR in PM epithelium was not associated with high cell-proliferation (assessed by Ki67), which would be expected to be present with classical ER α mediated epithelial action. Short term treatment with testosterone was associated with low proliferation index in normal PM women (Zang et al., 2008). Consistent with *in vitro* studies (Tuckerman et al., 2000; Berg et al., 2015), this may indicate a direct androgen driven induction of

AR, resulting in an inhibition of PM epithelial proliferation. Taking into account that stromal AR has been described to have an antiapoptotic role (Marshall et al., 2011), AR seems to have a cell specific function.

EHA is a premalignant condition (Lacey et al., 2008), with molecular aberrations (Steinbakk et al., 2011) and morphological changes that are typical for unopposed oestrogenic activity. Consistent with previous reports, the epithelial expression of AR in EHA was higher than in that of PP endometrium (Ito et al., 2002), whereas the proliferation index of EHA did not differ from that of PP endometrium. Notably, compared with PM endometrium, AR scores were relatively lower in EHA, with a significantly high Ki67 confirming oestrogen driven cell proliferation.

The available data on steroid receptor expression in EC subtypes are largely confined to endometrioid EC focusing on ER α , ER β and PR (Deligdisch et al., 2000; Kounelis et al., 2000; Collins et al., 2009). Traditionally type II ECs are considered to be hormonally-independent; there are only limited reports of ER and PR expression (Alkushi et al., 2010; Mhaweck-Fauceglia et al., 2013), with no previous data on AR and ER β expression in serous and clear cell carcinomas and carcinosarcomas (Hapangama et al., 2015). However, recent reports indicate that both type I and II ECs have similar risk factors (Setiawan et al., 2013). Studies describing AR expression in endometrioid EC are scarce and inconsistent. Horie et al. (1992) reported positive AR expression in 4 Grade 2 EC samples. In our study, most LGECs expressed AR (protein and transcript) and the LGEC immunoscores did not significantly differ from those of PM controls. Grade 3 endometrioid EC, however, expressed lower AR immunoscores consistent with the previous report (Ito et al., 2002). A conflicting study has reported the loss of AR in 72% of ECs (Sasaki et al., 2000), although, in that particular study, the specific histological type was not described for all samples. In agreement with our results, others have reported downregulation of PR in non-endometrioid HGEC (Alkushi et al., 2010). Interestingly, in comparison with LGECs, HGEC showed a significant decline in the expression of both PR and AR proteins, whereas the decrease in ER α was not significant. This was particularly evident in clear cell

carcinoma; although conflicting reports have shown a reduction of ER (without distinguishing ER α or ER β subtypes) in clear cell EC (Mhawech-Fauceglia et al., 2013; Hoang et al., 2014). Differences in sample size, methodology (antibodies and immuno-analysis) are likely to be the explanation for this discrepancy. From these results, it is tempting to speculate that even in presumed “hormonally independent” non-endometrioid HGEC, ER α and ER β receptors were expressed relatively abundantly (with concomitant loss of AR and PR), resulting in possible unopposed oestrogenic activity. In agreement with our data, the recent TCGA data also suggest AR expression to be a feature of LGECs with better prognosis (Kandoth et al., 2013). Furthermore, it was interesting that although ESR1 (ER α) and PGR (PR) gene mutation were seen in endometrial cancers with favourable outcome, AR mutations were not identified in the large TCGA dataset, suggesting the therapeutic potential of AR receptor modulators in EC.

The expression of AR in metastatic lesions was also examined and we observed, for the first time, a significant increase in AR immunoexpression in the metastatic EC tumours compared with matched primary tumours. Moreover, 60% of these lesions showed loss of PR, consistent with previous reports (Tangen et al., 2014). Previous studies have reported the expression of oestrogen receptors, ER β in particular in EC metastases (Sakaguchi et al., 2002). Although molecular and biological characteristics of macro-metastases are not well defined, low PR expression may explain the poor clinical response observed in a majority (66%) of patients with recurrent or metastatic tumours to progesterone treatment (Fiorica et al., 2004). The re-emergence of AR expression in these lesions, however, produces a possible novel adjuvant therapeutic opportunity.

Unlike breast cancer, the literature on the prognostic value of ER α , ER β , ER α /ER β ratio and PR in EC is inconsistent, probably owing to methodological variations (Takama et al., 2001; Fujimoto et al., 2002; Shabani et al., 2007; Jongen et al., 2009; Zannoni et al., 2013). In the current study, the immunoexpression of AR and PR was associated with longer DFS as well as CSS and OS of patients with EC and AR prediction of tumour relapse was independent of other prognostic

indicators. Consistent with our results, a recent study has reported the impact of AR expression in endometrioid ECs on the DFS of the patients; however, AR was not recognised as an independent indicator of patient outcome (Tanaka et al., 2015). Conversely, in a pooled study of 696 samples, PR was identified as a predictor of favourable survival with the combined HRs (95 % CI) of PR for OS, 0.63 (0.56–0.71), CSS, 0.62 (0.42–0.93) and DFS, 0.45 (95 % CI, 0.30–0.68) (Zhang et al., 2015). Intriguingly, the concurrent loss of both AR and PR was associated with a shorter DFS, CSS and OS. Moreover, loss of AR and PR correlated positively with unfavourable clinicopathological parameters that predict poor clinical outcomes such as high grade, deep myometrial invasion and cervical stromal involvement and advanced FIGO stages. AR can be activated by progesterone, which is the main hormonal therapy in EC, and subsequently may mediate inhibition of cell proliferation (Hackenberg and Schulz, 1996). Further, emerging evidence indicates that androgens, unlike progestin, may induce PR expression (Park et al., 2014). Therefore, besides the prognostic significance, expression data on AR and PR may be a useful clinically as a potential therapeutic target to tailor adjuvant hormonal therapy for intermediate and higher risk EC. Consistent with previous studies, we have also shown that the loss of ER α and the low ratio of ER α /ER β were significantly associated with shorter OS (Takama et al., 2001; Zannoni et al., 2013).

Chapter Four

The Prognostic Role of MIPs

4.1 Introduction

Endometrial cancer is the commonest gynaecological malignancy yet is perceived by many as a relatively indolent cancer, due to the early detection and subsequent surgical excision of the uterus. Despite the fact that surgical removal of endometrial cancer is associated with a favourable outcome, 2-15% of early stage and 50% of advanced stage endometrial cancer (EC) will eventually recur (Salani et al., 2011). Clinicopathological classification of EC into low, intermediate and high risk has been criticised to be of limited specificity in stratification of patients for postsurgical management (Koskas et al., 2016). Although a recent molecular classifier has proposed a four cluster classification that can be integrated with clinicopathological classifier to improve patient stratification (Talhok et al., 2015), reliable markers for predicting response to therapy or tumour recurrence are lacking. Furthermore, the discovery of biomarkers for early detection of endometrial cancer and identifying patients with atypical hyperplasia (EHA) who are at risk of progressing into malignant disease remains challenging.

Metastasis inducing proteins (MIPs) have been proposed as prognostic indicators in a wide range of human malignancies (Barracough et al., 2009; Abeloos et al., 2011; Mishra et al., 2012). The attribution of the family of S100 proteins to migratory properties and metastasis has been comprehensively reviewed elsewhere (Boye and Gunhild, 2010). Possible involvement of S100A4 and to lesser extent S100P in epithelial to mesenchymal transition has also been described and gaining interest (Teng et al., 2007; Techasen et al., 2014; Xu et al., 2014; Zhai et al., 2014; Hsu et al., 2015); S100A4 as a marker for poor prognosis and recurrence in EC has recently been reported (Chong et al., 2014). Yet, the change in the expression pattern of these two proteins throughout the different stages of endometrial neoplastic transformation and progression has not yet been fully described.

Parallel to S100 proteins, AGR2 has also been reported as a prognostic marker in several hormonally regulated cancers such as breast (Liu et al., 2005), prostate

(Kani et al., 2013) and ovary (Edgell et al., 2010). In addition to its involvement in drug resistance and metastatic growth (Hengel et al., 2011; Gray et al., 2012), AGR2 overexpression results in extracellular secretion and can be detected in extracellular fluids (Clarke et al., 2015). Hence it represents a compelling biomarker for cancer detection and/or follow-up. While many ECs are hormone responsive, the pattern of AGR2 expression has not been explored and a possible role in endometrial carcinogenesis has yet to be described.

In this chapter, protein and mRNA expression of S100A4, S100P and AGR2 are described individually in all subtypes of human endometrial cancer tissue and compared to normal (pre and post-menopausal) and premalignant EHA endometrium. Further, the expression patterns of these MIPs are described for the first time in metastatic endometrial lesions and compared to their matched primary tumours in an attempt to illustrate the consequences of tumour progression on neoplastic cell phenotype. Finally, the feasibility of utilising AGR2 as a biomarker is also examined.

4.2 Research questions

1. Are the metastasis inducing proteins S100A4, S100P and AGR2 differentially expressed in endometrial cancer and do they correlate with steroid receptor expression and proliferation index?
2. Do metastatic lesions express MIPs in a pattern similar to matched primary tumours?
3. Can each of the MIPs individually predict EC recurrence and cancer specific death?
4. Will a panel of three MIPs improve the individual protein prediction of tumour progression and patient survival?
5. Is AGR2 secreted by the EC cells and can it be used as a biomarker for detection or predicting prognosis of EC?

4.3 Methods

4.3.1 Patients and samples

A total of 161 formalin fixed paraffin embedded human endometrial tissue samples were included as detailed in Table 4.1. Eighteen frozen endometrial biopsies; 5 PM, 5 LGEC and 8 HGEC were used for RNA extraction and RT-qPCR downstream.

Table 4.1 Patient groups and demographics.

Study groups	No	Age* (years)	BMI* kg/m²
Proliferative phase	16	39 (30-49)	26.7 (18-46)
Postmenopausal	15	64 (52-79)	26 (22-38)
Endometrial hyperplasia	14	55 (48-73)	29.90 (24-57)
No atypia	4	55(50-62)	27.9 (24-53)
With atypia	10	55 (48-73)	30.10 (24-57)
Endometrial cancer	100	67 (41-89)	30.7 (20-54)
LGEC	50	64 (41-84)	30.8 (22-54)
Grade1	30	64 (51-84)	32 (22-53)
Grade2	20	64 (41-77)	29 (22-54)
HGEC	50	70 (51-89)	29.6 (20-43)
Grade3	15	69 (51-83)	26.7 (22-43)
Serous	8	73 (64-82)	29.8 (25-35)
Clear cell	12	69 (52-82)	29.9 (25-32)
Carcinosarcoma	15	78 (59-89)	24.2 (20-37)
Metastatic lesions	16	68 (41-89)	-
* Data expressed as median (range)			

4.3.2 IHC

Included samples were immunostained as described in 2.7.1.1. Antigen retrieval buffer, primary Ab details are all summarised in Table 4.2.

Table 4.2 Primary antibodies and their immunohistochemistry conditions.

Primary Ab	Type	Clone	Supplier (#number)	HIAR* (min)	Dilution	Incubation conditions	
						Time (hour)	Temp (°C)
S100A4	Polyclonal		DAKO ¹	2	1:200	20	4
AGR2	Monoclonal	EPR3278	Abcam ²	2	1:1500	20	4
S100P	Monoclonal	16	BD (610307)	2	1:100	2	18
PanCK	Monoclonal		Sigma ³	2	1:6000	20	4
CD68	Monoclonal	PG-M1	DAKO	2	1:50	20	4
CD45	Monoclonal		DAKO	2	1:50	20	4
CD14	Monoclonal		Biol Sci ⁴	2	1:50	20	4

*Heat induced antigen retrieval by pressure cooking in citrate buffer pH 6 (Hapangama et al, 2012). Ely, Cambridgeshire, UK (¹); Cambridge, UK (²); UK (³); UK (⁴).

4.3.3 IHC analysis

The expression of S100A4 and S100P in epithelial cells nuclei and cytoplasm were semi-quantified separately using *Hscore* as previously described in 2.7.1.2. The expression of S100A4 in stromal compartment, immune cells and blood vessels was also semi-quantified by *Hscore* but with estimating the proportion of positive cells rather than cell counting.

Epithelial cytoplasmic AGR2 expression was semi-quantified using a quick score as previously described in 2.7.1.2. AGR2 quick score was optimised as follows;

The percentage of positive AGR2 stained target cells in each of four intensity categories (0= no staining, 1= weak staining, 2= moderate staining and 3= strong staining) were initially estimated in 30 randomly selected endometrial samples. *Hscore* was then calculated using the formula described by McCarty (1986):

$$Hscore = \sum p_i(i+1)$$

where i represents the intensity of the staining and P_i varies from 0 to 100%. We then proposed three four tier quick scores where the proportions of positive cells were:

1. 1%-25%=1, 26%-50%=2, 51%-75%=3 and >76%=4
2. 1%-10% =1, 11%-30% =2, 31%-50% =3, and >50%=4.
3. 1%-10% =1, 11%-20% =2, 21%-40% =3, and >40%=4.

The score for the proportion of positive cells was then multiplied by the staining intensity categories and added to give a final score out of 12. Total scores generated from each proposed scoring set for the selected samples were subsequently correlated with total H score for the same samples. The Quick score categories shown above in number 2 showed the best correlation with H score, and were therefore used for quantification of AGR2 in this study.

4.3.4 PCR

Messenger RNA was amplified as described in chapter 2.8.5.2 using the primers listed in Table 4.3 and quantified relative to the geometric mean of appropriate reference genes *YWHAZ* and *PPIA* as described by (Jacob et al., 2013; Romani et al., 2014). For each target and reference gene, a standard curve was generated and primer efficiency was determined (Figure 4.1A). Products were resolved after the first amplification on agarose gel (Figure 4.1B) to confirm the specificity of the primers and rule out any off target amplicons.

Table 4.3 Primer sequences used for RT-qPCR amplification.

Primer	Sequence	Amplicon	Efficiency	Reference
AGR2	5-ATTGGCAGAGCAGTTTGTCC-3	179	98.8%	(Hu et al. 2012)
	5-GAGCTGTATCTGCAGGTTCTG-3			
S100A4	5-AGCTACTGACCAGGGAGCTG-3	138	96.2%	(Schneider et al. 2007)
	5-TGCAGGACAGGAAGACACAG-3			
S100P	5-GATGCCGTGGATAAATTGCT-3	141	99.9%	(Ohuchida et al. 2006)
	5-AGGGCATCATTTGAGTCCTG-3			
YWHAZ	5-CGTTACTTGGCTGAGGTTGCC-3	69	91.1%	(Marullo et al. 2010)
	5-GTATGCTTGTGTGACTGATCGAC-3			
PPIA	5-AGACAAGGTCCCAAAGAC-3	118	96.6%	(Jacob et al. 2013)
	5-ACCACCCTGACACATAAA-3			

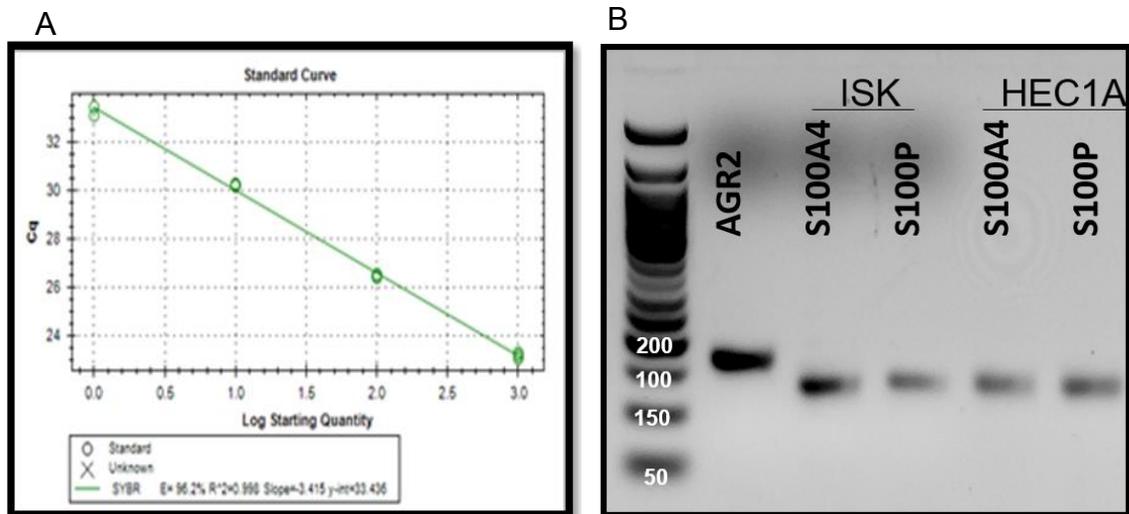


Figure 4.1 The efficiency and specificity of RT-qPCR primers. (A) An example of standard curve showing assay efficiency, precision and slope. (B) Image of agarose gel electrophoresis showing the specific band for each target.

4.3.5 ELISA

AGR2 protein in the serum and uterine washes from normal PM and EC patient was detected using commercially available AGR2 ELISA kit (SEC285Hu, Uscon Life Science Inc., China) as previously described 2.7.2. The following validation assays were performed to examine the effect of several factors such as sample matrix and heterophilic antibodies which might interfere with assay accuracy:

4.3.5.1 Spike/ recovery

One pg of ELISA standard or recombinant human AGR2 protein (rhAGR2) were “spiked” into serum samples with high and low AGR2 levels, both of which were within the published linear range of the standard curve. Samples were run along with serially diluted standards as per protocol. Spike/recovery was calculated following the equation.

$$\% \text{ Recovery} = \frac{\text{Observed} - \text{Neat}}{\text{Expected}} \times 100$$

Observed = Spiked sample value

Neat = Unspiked sample value

Expected = Amount spiked into sample

4.3.5.2 Linearity

Linear dilution is a critical experiment to validate the specificity and the accuracy of an assay. Non-linear dilution would indicate that a sample component is interfering with accurate detection of a specific analyte at a given dilution. Three dilutions; neat, 1:2, 1:4 of the rhAGR2 spiked sample were used to assess the kit's linearity.

4.3.5.3 Precision

Intra-assay precision was assessed by testing a sample with a high level of AGR2 three times in the same plate.

Inter-assay precision was assessed by testing three samples with low, middle and high AGR2 on three different plates.

CV (%)= SD/ mean x100

4.3.5.4 Specificity

Because AGR2 shares 72% homology with AGR3; recombinant AGR3 was also spiked into the samples to test kit specificity to AGR2.

4.3.5.5 Statistics

Descriptive data were presented in median and range. Statistical analyses were performed in IBM SPSS 22 as previously described in 2.9. Graphs were plotted using graph pad prism 5.

4.4 Results

4.4.1 Patient demographics

As Table 4.1 demonstrates, patients in proliferative phase were significantly younger than those in menopause ($P < 0.0001$) and those who developed EC ($P < 0.0001$). Although 10/12 (83%) of hyperplastic lesions were in the background of endometrial cancer, those patients were significantly younger than other EC patients ($P = 0.024$). This difference was even more pronounced when compared with the high grade cancer group (HGEC, $P = 0.001$). Within EC patients, all participants were postmenopausal; while women with HGEC were significantly older than those with low grade cancers (LGEC, $P = 0.02$). Statistical analyses were adjusted for multiple testing.

LGEC and EHA had a tendency to occur in more obese women compared with women in proliferative phase (PP, $P=0.02$, $P=0.03$ respectively), however, significance was lost after adjusting for multiple testing ($P=0.24$, $P=0.30$ respectively). BMI ≥ 30 was also observed more frequently in LGEC compared with HGEC ($P=0.04$, Adj $P=0.367$) suggesting obesity was a feature of LGEC.

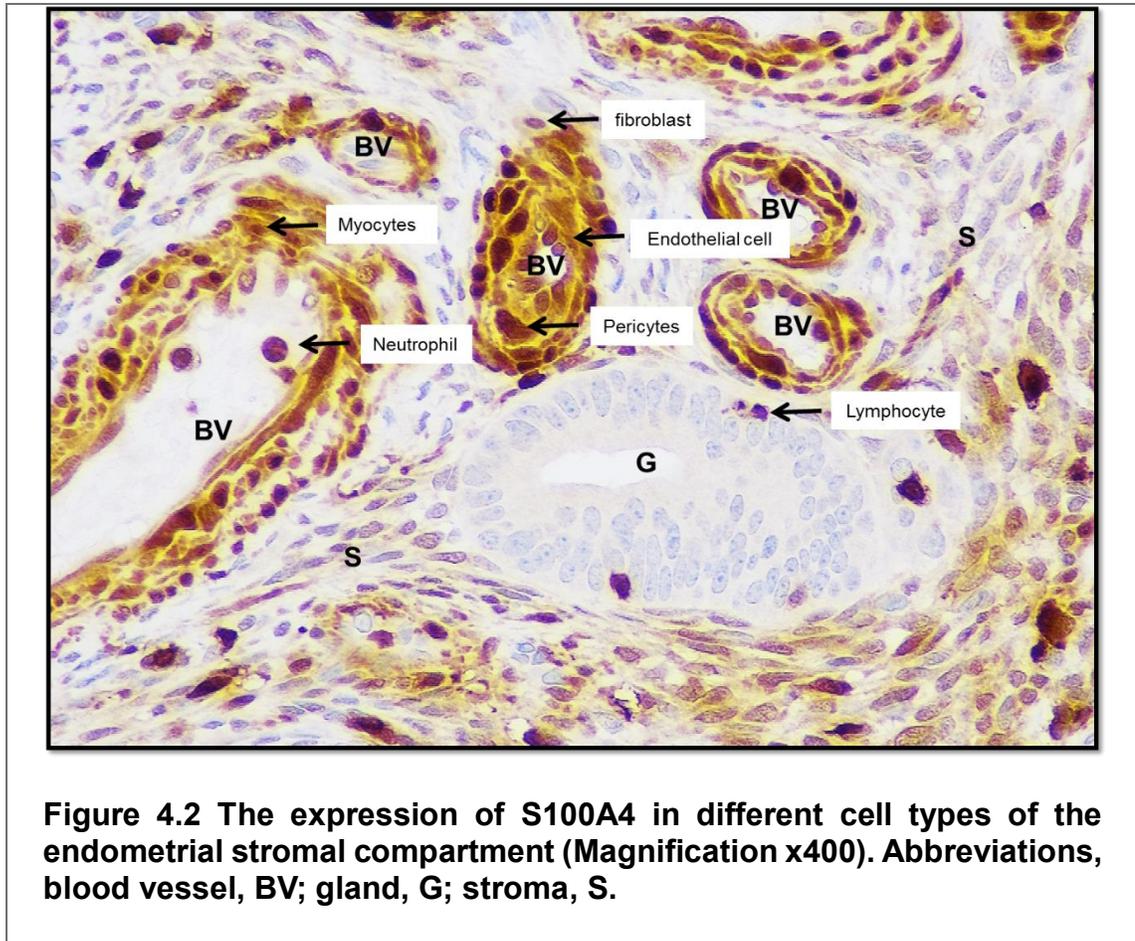
4.4.2 S100A4

4.4.2.1 IHC results

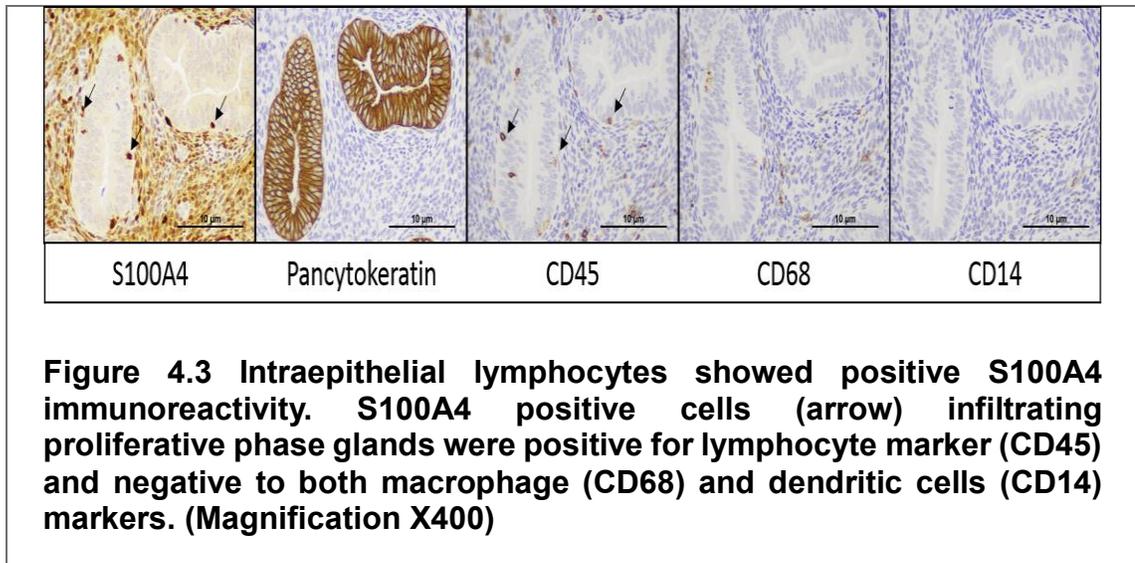
S100A4 protein was observed in the epithelial and stromal compartments of the human endometrium as well as blood vessels and inflammatory cells (lymphocytes, leukocytes, and macrophages). Epithelial staining was semi-quantified in the nuclei and the cytoplasm separately whereas stromal staining was scored in the whole compartment (fibroblasts, endothelial and inflammatory cells). Nuclear S100A4 was significantly associated with cytoplasmic S100A4 expression (Spearman correlation, $r=0.901$, $P<0.0001$)

4.4.2.1.1 S100A4 is a feature of normal endometrial stroma

S100A4 protein was predominantly expressed in the stroma of the normal pre and postmenopausal endometrium. Nuclear and cytoplasmic expression were observed in all cell types of the stromal compartment; including fibroblasts, lymphocytes, neutrophils, macrophages and in endothelial cells as shown in Figure 4.2. Significant upregulation of stromal S100A4 was observed after menopause ($P= 0.007$, Figure 4.5d)



In some PP endometrial glands, S100A4 was immunolocalised in single cells. Serial sections have characterised these cells as CD45+, CD68-, CD14- indicating that PP glands were infiltrated by S100A4 positive lymphocytes whereas all epithelial cells were immunonegative as shown in Figure 4.3. Similarly, intraepithelial lymphocytes (CD45+) were S100A4 positive in PM endometrial glands; although, weak nuclear and cytoplasmic epithelial staining (CD45-) was also observed in postmenopausal (PM) endometrial glands.

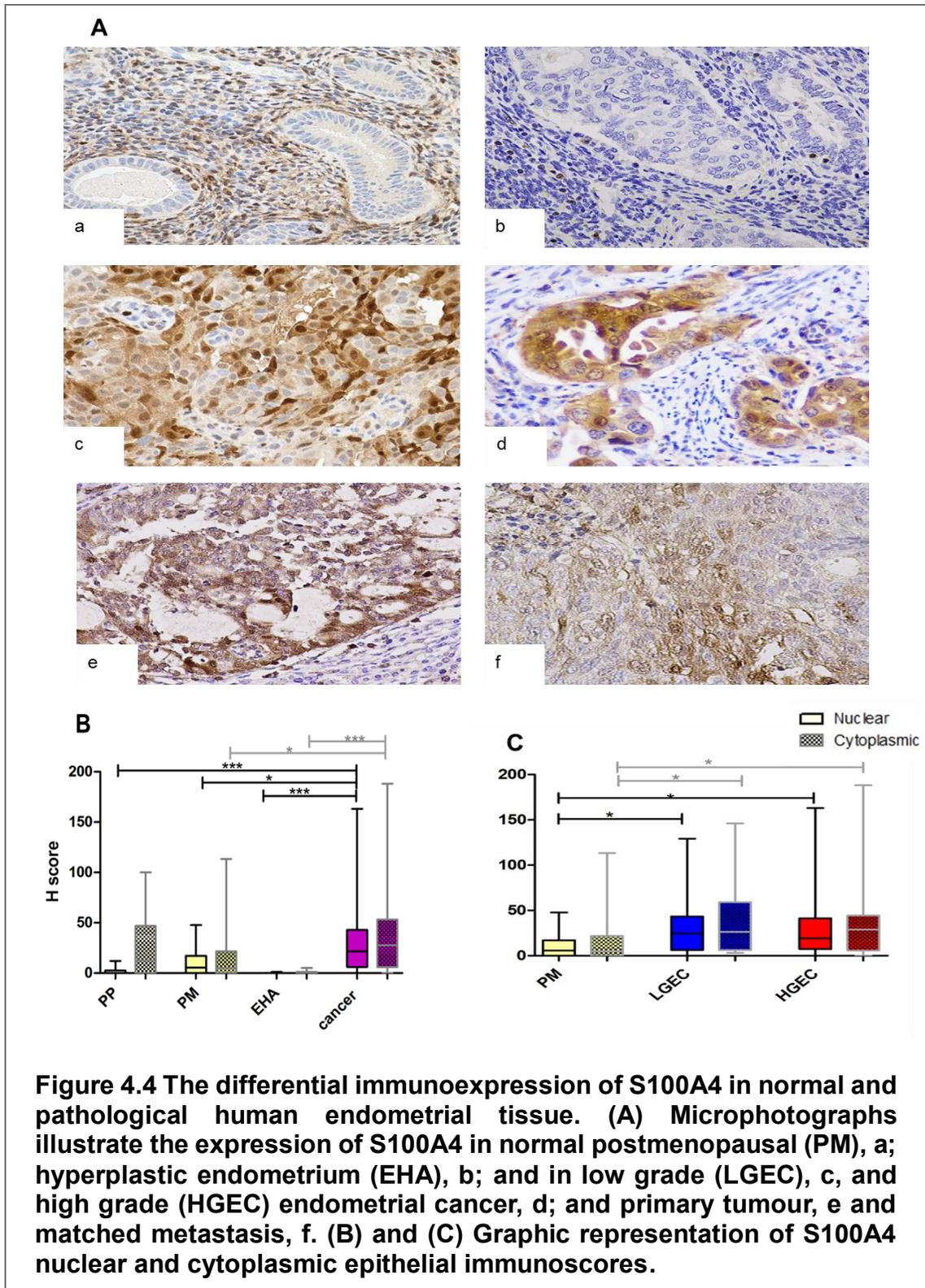


4.4.2.1.2 Hyperplastic endometrium does not express S100A4

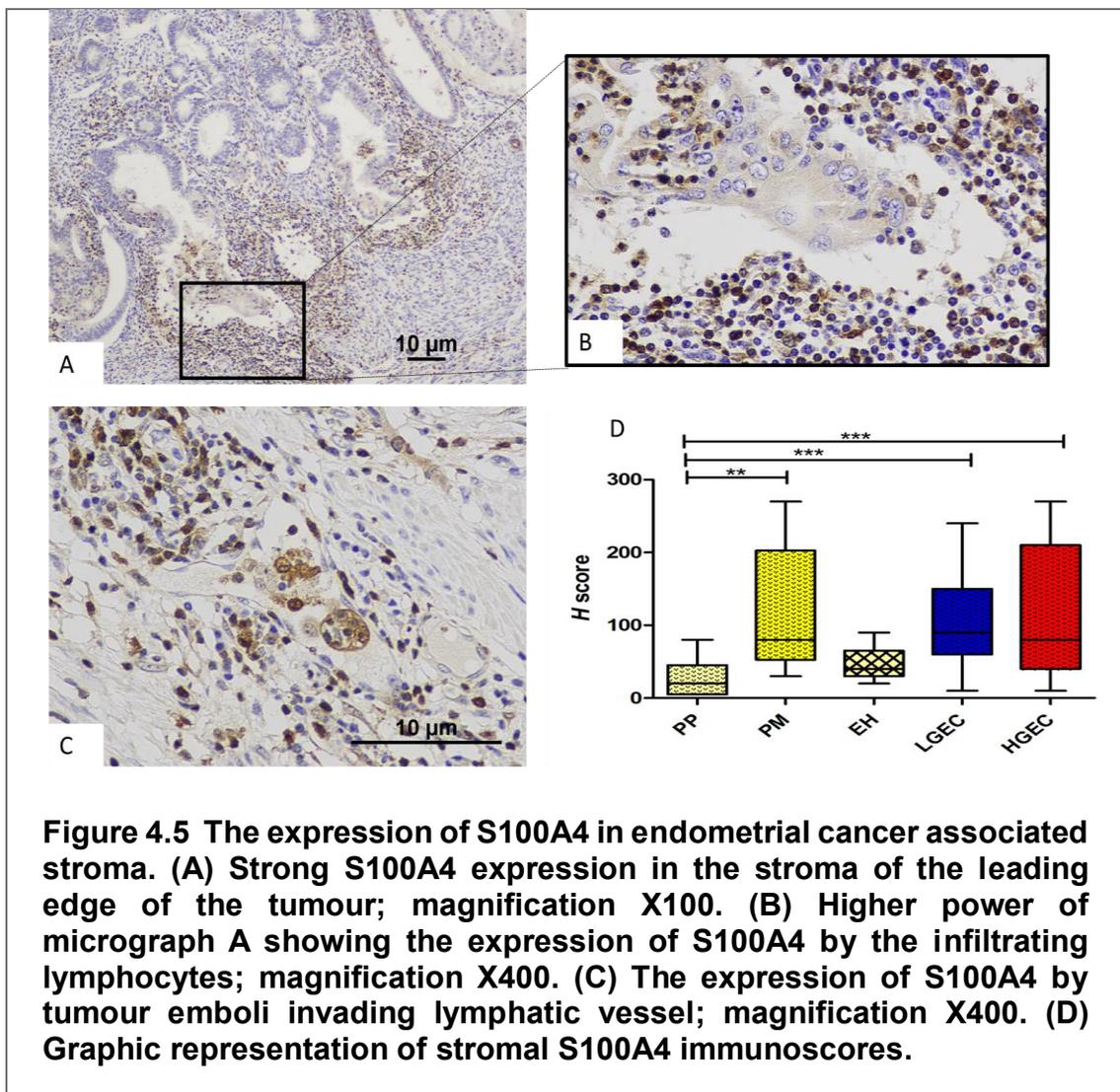
Generally, hyperplastic endometrial epithelium (with or without atypia) were nonreactive for S100A4. Weak cytoplasmic reactivity was observed in sporadic epithelial cells of EHA samples and was significantly lower than that of LGEC ($P < 0.0001$) and HGEC ($P = 0.001$), Figure 4.4B. Nuclear S100A4 was completely absent in hyperplastic epithelium regardless of nuclear atypical changes, Figure 4.4A. S100A4 expression in the associated stroma was similar to that of PP (Figure 4.5d).

4.4.2.1.3 S100A4 is upregulated in endometrial cancer

As shown in Figure 4.4B, the expression of nuclear and cytoplasmic S100A4 were significantly higher in EC compared with normal epithelial cells (PP, nuclear $P < 0.0001$; PM, nuclear $P = 0.022$, cytoplasmic $P = 0.028$) and hyperplastic epithelial cells (nuclear and cytoplasmic, $P < 0.0001$). LGEC (nuclear, $P = 0.014$; cytoplasmic, $P = 0.013$) and HGEC (nuclear, $P = 0.031$; cytoplasmic, $P = 0.032$) expressed higher S100A4 than normal PM.

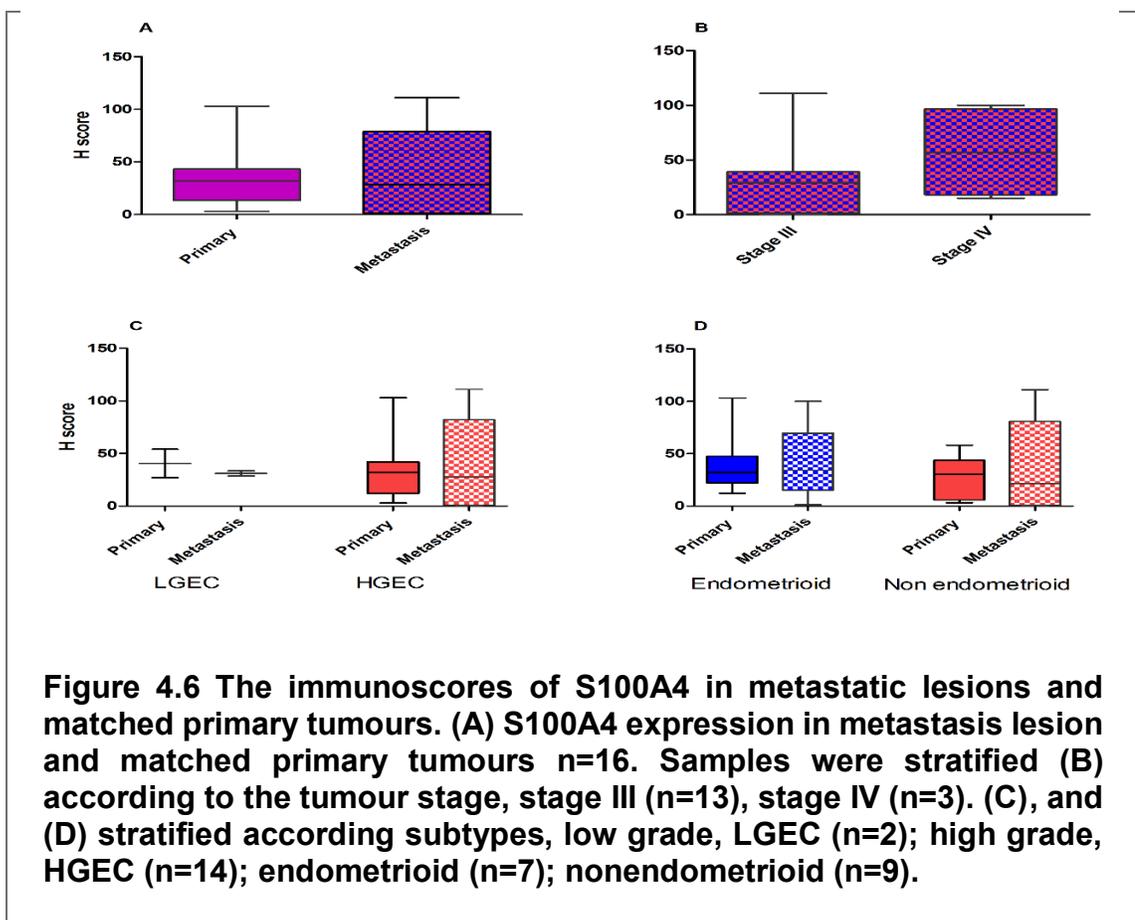


No difference in S100A4 reactivity was observed between LGEC and HGEC (Figure 4.4C). S100A4 expression in cancer associated stroma was similar to the stroma of the normal PM endometrium; and significantly higher than that of the PP endometrium ($P < 0.0001$, Figure 4.5d). This was principally due to infiltrating lymphocytes which showed strong immunoreactivity to S100A4; however, macrophages and fibroblast were S100A4 positive as well. Intriguingly, stroma in the leading edge of the tumour in most of the cases was associated with high expression for S100A4 as shown in Figure 4.5.



4.4.2.1.4 Metastatic lesions of endometrial cancer maintain S100A4 expression

S100A4 was observed in the nuclei and the cytoplasm of the metastatic EC cells (Figure 4.4A). Similar to primary tumours nuclear and cytoplasmic S100A4 expression were strongly correlated in metastatic lesions ($r=0.918$, $P<0.0001$). Out of 15 metastatic lesions, only one sample (6.6%) showed a complete absence of nuclear and cytoplasmic S100A4 expression, 4 (26%) showed reduced nuclear expression, whereas 10 (60%) showed comparable or increased nuclear S100A4 expression compared with matched primary tumours (Figure 4.6A).



There was no significant difference between S100A4 expression in the stroma associated with primary tumour and in the matched metastasis associated-stroma.

Interestingly, S100A4 expression seems to be higher in the distant metastatic lesions from stage IV compared with those from stage III as shown in Figure 4.6B, however, this trend did not reach statistical significance. When metastatic lesions were stratified according to the histology of the primary tumours to S100A4 in LG vs HG (Figure 4.6C) or endometrioid vs nonendometrioid (Figure 4.6D), the respective S100A4 expression in the metastatic lesions were not different from the expression of the matched primary.

4.4.2.1.5 S100A4 is associated with deep myometrial invasion

In order to assess the impact of S100A4 on common EC prognostic parameters (Table 4.4) EC was categorised according to the expression of S100A4, to either S100A4+ or S100A4-. Several cut-off points were employed for nuclear and cytoplasmic expression, with 5% (*Hscore*= 15) expression giving the best delineation of patients with the worst outcome. Since nuclear and cytoplasmic S100A4 were strongly correlated, nuclear expression was chosen to investigate the prognostic value of S100A4.

As shown in Table 4.4, positive S100A4 expression was associated with myometrial invasion; 73% of cases with deep myometrial invasion were S100A4+. Differential S100A4 expression was not observed with the other clinicopathological parameters or the expression of steroid receptors.

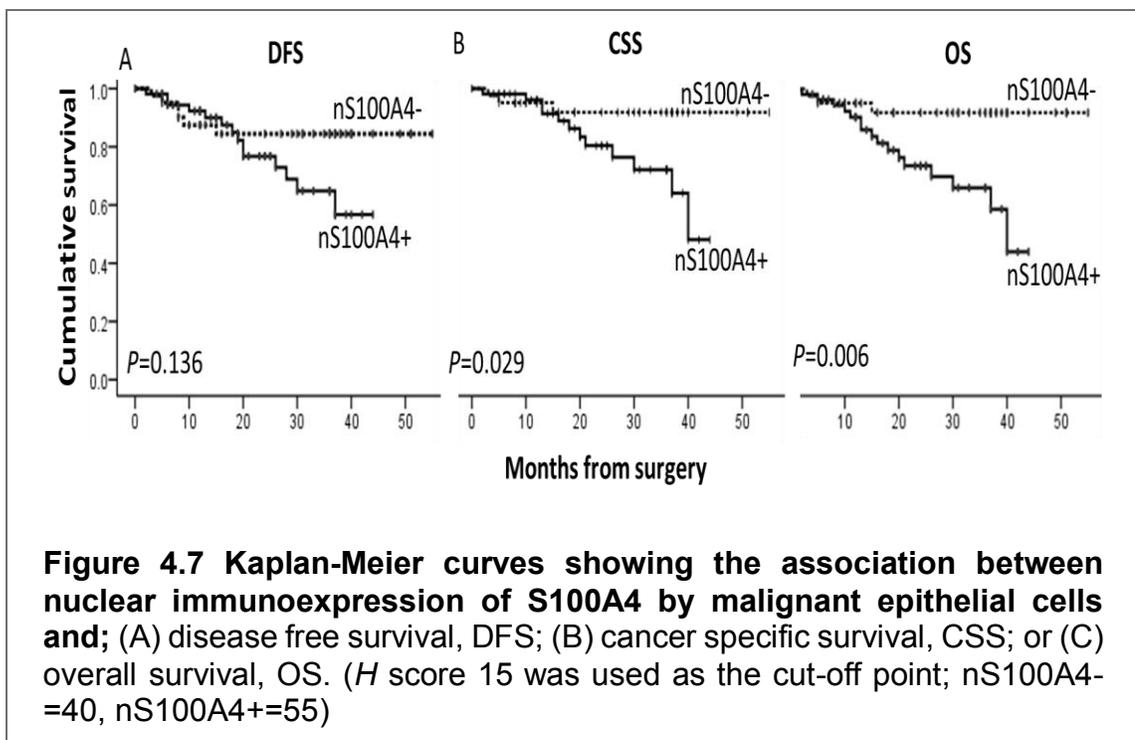
Table 4.4 The association of S100A4 with clinicopathological factors.

Variables	Total	S100A4				P
		-	%	+	%	
Age	≤65	100	20 (43)	27 (57)	0.766	
	>65		21 (40)	32 (60)		
BMI	<30	68	12 (38)	20 (62)	0.626	
	≥30		16 (44)	20 (56)		
Grade	LG	100	32 (54)	27 (46)	0.309	
	HG		18 (36)	32 (64)		
Stage	I-II	98	27 (44)	34 (56)	0.373	
	III-IV		13 (35)	24 (65)		
Myometrial invasion	<50	97	29 (51)	28 (49)	0.021	
	≥ 50		11 (28)	29 (72)		
Cervical invasion	-	97	30 (43)	40 (57)	0.602	
	+		10 (37)	17 (63)		
LVI	-	98	25 (43)	33 (57)	0.579	
	+		15 (37)	25 (63)		
LN	-	20	3 (37)	5 (63)	1	
	+		4 (33)	8 (67)		
Extra-uterine invasion	-	96	10 (32)	21 (68)	0.249	
	+		29 (45)	36 (55)		
AR	-	86	15 (47)	17 (53)	0.284	
	+		19 (35)	35 (65)		
PR	-	86	20 (47)	23 (53)	0.186	
	+		14 (33)	29 (67)		
ERα	-	86	4 (36)	7 (64)	1	
	+		30 (40)	45 (60)		
ERβ	-	86	0 (0)	1 (100)	1	
	+		34 (40)	51 (60)		
ERα/ERβ ratio	low	86	7 (30)	16 (70)	0.297	
	high		27 (43)	36 (57)		
Ki67	low	85	9 (38)	15 (63)	0.875	
	high		24 (39)	37 (61)		

Abbreviations: Lymphovascular space invasion (LVSI), Lymph node (LN).

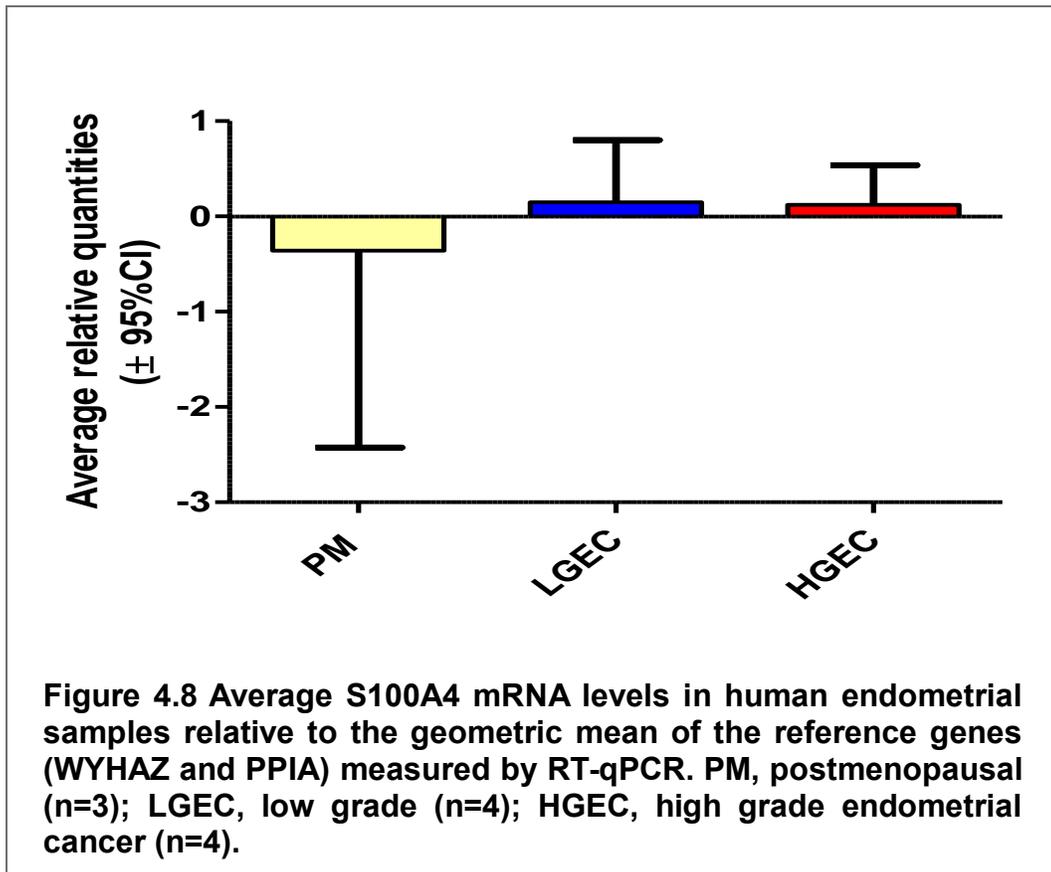
4.4.2.1.6 S100A4 is associated with shorter cancer specific and overall survival

The Kaplan-Meier curves (Figure 4.7B and C) showed that the proportion of patients with S100A4+ tumours who developed EC specific death was higher than those with S100A4- cancers (CSS, $P=0.029$) and the overall survival (OS) in S100A4+ was shorter ($P=0.006$). S100A4 expression did not show a difference with disease free survival (DFS, Figure 4.7A). When the Cox progression model was applied, univariate analysis showed that S100A4 expression was associated with poor CSS (HR=3.8, 95%CI=1.05-13.6, $P=0.041$) and OS (HR=4.8, 95%CI=1.4-16.8, $P=0.013$). Multivariate cox regression analysis did not identify S100A4 as an independent prognostic indicator of CSS or OS.



4.4.2.2 The mRNA level of S100A4 in human endometrial tissue

The overall transcriptional activity of S100A4 in human endometrial tissue was similar to protein immunoreactivity; although the correlation between S100A4 mRNA levels and the nuclear localisation of the protein (immunoscore) was poor ($r=0.21$, $P=0.616$). Both LGEC and HGEC express higher S100A4 mRNA compared with the normal postmenopausal endometrial samples (Figure 4.8). The difference between groups did not reach statistical significance.



4.4.3 S100P

4.4.3.1 IHC results

S100P protein was expressed in the nuclei and the cytoplasm of both epithelial and stromal compartments of the endometrium. Positive S100P immunoexpression was also observed in the endometrial blood vessels and immune cells. Nuclear S100P immunostaining was not always associated with cytoplasmic expression ($r= 0.47$, $P<0.0001$)

4.4.3.1.1 S100P is expressed in the cytoplasm of healthy endometrium epithelium

Generally, the expression of S100P protein in the healthy endometrium was scanty. It was predominantly expressed in the glandular epithelial cells as a weak to moderate intensity perinuclear stain, however, discrete punctate cytoplasmic staining was also seen in a few samples (Figure 4.9). Unlike S100A4, stromal S100P was limited to a few fibroblasts and neutrophils.

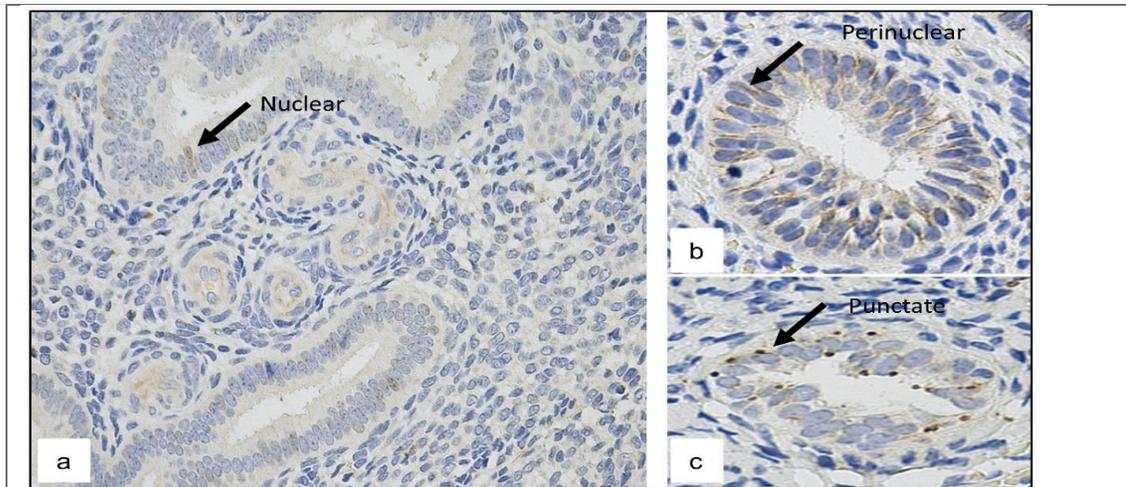
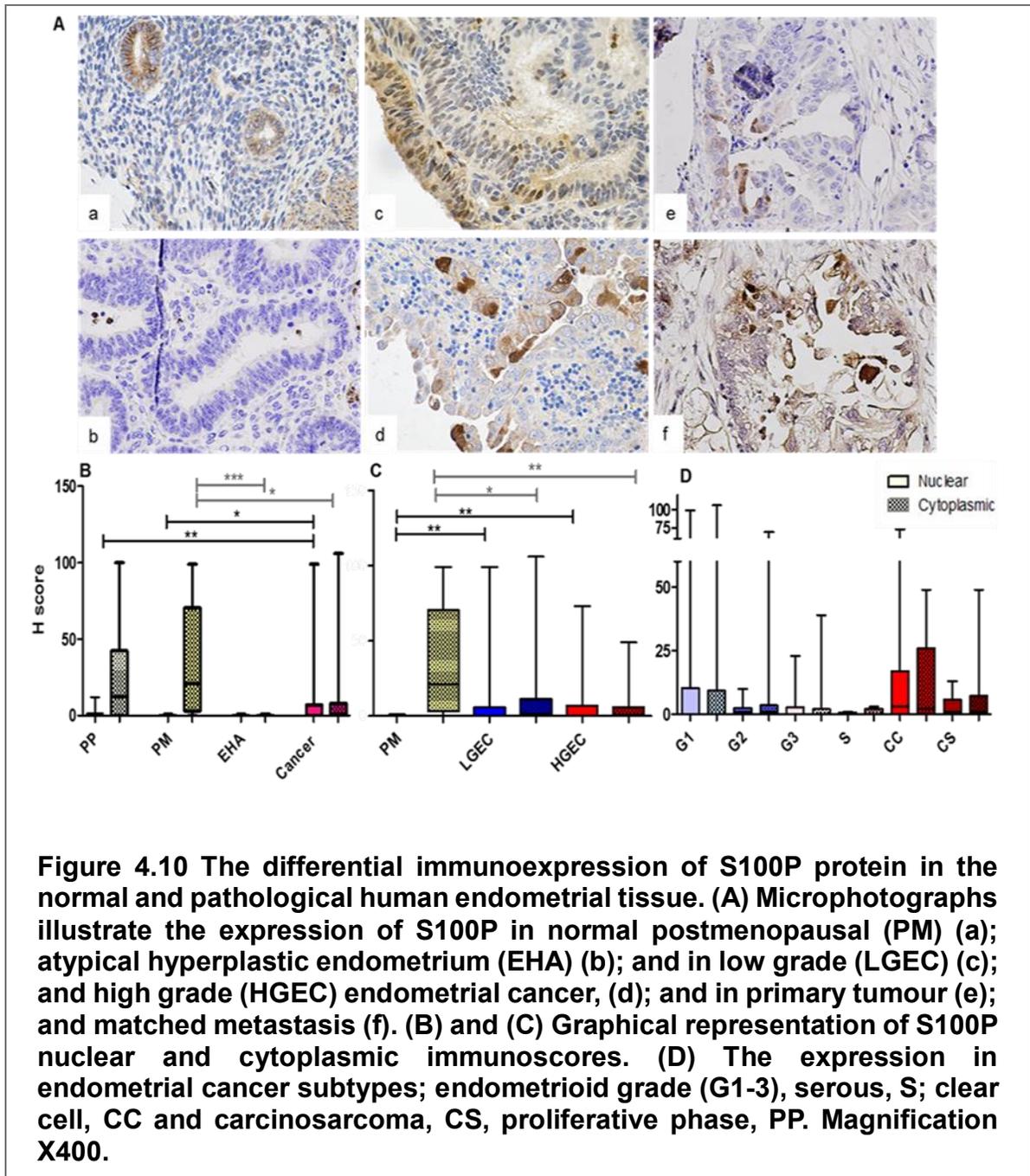


Figure 4.9 The cellular localisation of S100P protein in the glandular epithelium of proliferative phase endometrium. A representative micrograph illustrating a weak nuclear staining, Magnification x200 (a); or the cytoplasmic (perinuclear) (b); punctate pattern (c). Magnification x400



4.4.3.1.2 Hyperplastic endometrium does not express S100P

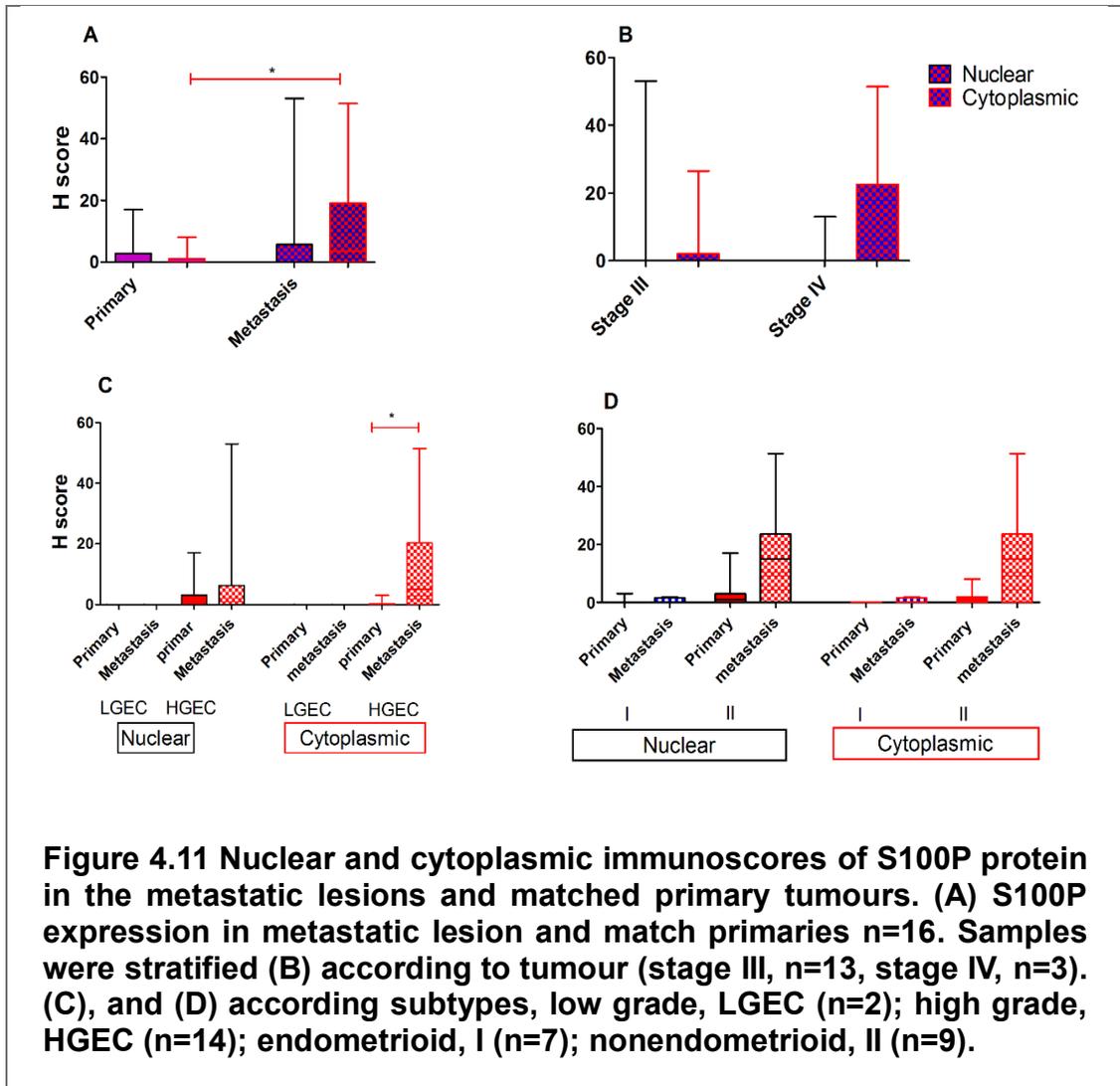
S100P was not expressed by EH with or without cytological atypia. There was a complete absence of both nuclear and cytoplasmic S100P immunoreactivity in the hyperplastic epithelial cells. Stromal expression was limited to the sporadic fibroblasts and inflammatory cells, Figure 4.10A and B.

4.4.3.1.3 S100P is upregulated in malignant endometrium

S100P expression was observed in 60% of tumours from our EC cohort. These constituted 31/50 (62%) of LGEC and 29/50 (58%) of HGEC. The pattern of S100P expression was focal and patchy limited to a small population of tumour cells. Malignant endometrial cells showed a significant upregulation of nuclear S100P compared with the healthy PM controls ($P=0.007$; Figure 4.10 A and B). This was accompanied by a significant loss of cytoplasmic immunoreactivity ($P=0.036$). When EC was stratified according to tumour grade, both LGECs and HGECs expressed similar immunoscores which were significantly higher than those of the PM endometrium ($P=0.040$, $P=0.043$ respectively, Figure 4.10C). Notably, among EC subtypes, clear cell carcinomas showed the highest S100P expression scores (Figure 4.10D). In the stromal compartment of ECs, immunoreactivity to S100P was similar to that of the normal endometrium.

4.4.3.1.4 Cytoplasmic S100P is upregulated in metastatic lesions of endometrial cancer

Eight out of 15 metastatic lesions (53%) showed immunoreactivity to S100P which tended to be higher than the expression scores in the matched primary tumours (Figure 4.10A). Unlike the primary tumours, not only nuclear expression was higher in the metastatic lesions, but the cytoplasmic S100P also showed a significant increase ($P=0.008$); this increase in S100P was even more prominent in lesions from stage IV cancers (Figure 4.11A and B). The two metastatic lesions from LGEC were completely negative for S100P whereas grade 3 endometrioid immunoreactivity for S100P was comparable to that of matched primary cancer sample. Metastatic lesions from non-endometrioid ECs showed higher S100P expression (both nuclear and cytoplasmic) compared with the matched primary tumour (Figure 4.11 C and D).



4.4.3.1.5 The association between S100P immunostaining and clinicopathological parameters

ECs were categorised according to the expression of S100P in order to investigate its relationship with clinicopathological parameters (Table 4.5). Samples with no observed S100P (H score <1) of S100P were considered as S100P- and those with staining (H score ≥ 1) were deemed to be S100P+. S100P+ was observed more often in EC patients younger than 65 years particularly when considering the cytoplasmic expression ($P=0.041$). ER α was positive in more than 90% of the tumours with S100P+ which showed a significant association with the nuclear S100P expression ($P=0.022$). Cervical stromal invasion ($P=0.042$) and LVSI ($P=0.056$) were inversely associated with cytoplasmic S100P+ suggesting that cytoplasmic S100P associated with good prognostic indicators. S100P showed a different expression pattern in serous EC compared with other histological sub-types; therefore, association with clinicopathological parameters were re-assessed after excluding serous cancer cases from the analysis. The positive associations with age (nuclear S100P+, $P=0.094$; cytoplasmic S100P+, $P=0.035$) and ER α (nuclear S100P+, $P=0.025$; cytoplasmic S100P+, $P=0.080$) were maintained in the absence of serous EC; whereas the inverse associations of cytoplasmic S100P with cervical stromal invasion ($P=0.098$) and (LVSI $P=0.196$) were lost. There was no significant association observed with S100P and the other clinicopathological parameters that were examined as shown in Table 4.5

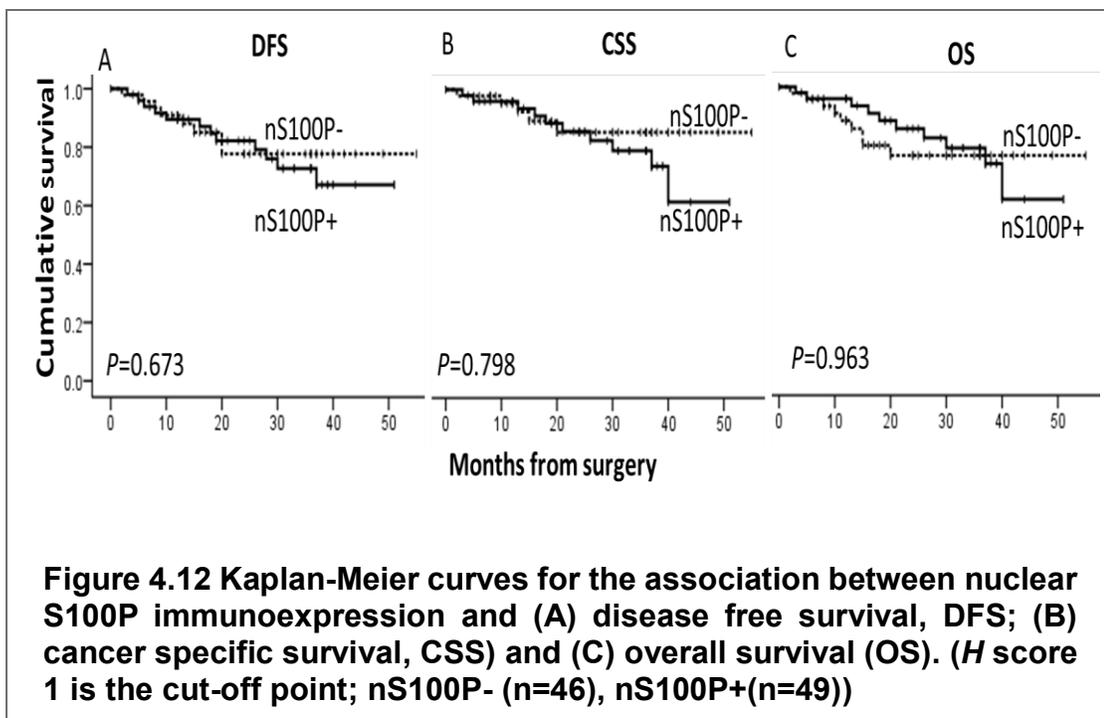
Table 4.5 The association between S100P immunostaining (nuclear and cytoplasmic) and clinicopathological factors.

Variables	Total	Nuclear S100P			Cytoplasmic S100P			
		- (%)	+ (%)	P	- (%)	+ (%)	P	
Age	<=65	100	19 (40)	28 (60)	0.115	17 (36)	30 (64)	0.041
	>65		30 (57)	23 (43)		30 (57)	23 (43)	
BMI	<30	68	17 (53)	15 (47)	0.158	17 (53)	15 (47)	0.1
	>=30		13 (36)	23 (64)		12 (33)	24 (67)	
Grade	LG	100	23 (46)	27 (54)	0.689	23 (46)	27 (54)	0.841
	HG		26 (52)	24 (48)		24 (48)	26 (52)	
Stage	I-II	98	29 (47)	32 (53)	0.715	28 (46)	33 (54)	0.601
	III-IV		19 (51)	18 (49)		19 (51)	18 (49)	
Myometrial invasion	<50	97	25 (44)	32 (56)	0.28	23 (40)	34 (60)	0.215
	≥ 50		22 (55)	18 (45)		22 (55)	18 (45)	
Cervical invasion	-	97	30 (43)	40 (57)	0.075	28 (40)	42 (60)	0.042
	+		17 (63)	10 (37)		17 (63)	10 (37)	
LVSI	-	98	24 (41)	34 (59)	0.116	22 (38)	36 (62)	0.056
	+		23 (57)	17 (43)		23 (58)	17 (42)	
LN	-	20	4 (50)	4 (50)	1	4 (50)	4 (50)	1
	+		5 (42)	7 (58)		5 (42)	7 (58)	
Extrauterine metastasis	-	96	17 (55)	14 (45)	0.426	17 (55)	14 (45)	0.28
	+		30 (46)	35 (54)		28 (43)	37 (60)	
AR	-	86	13 (41)	19 (59)	0.314	13 (41)	19 (59)	0.498
	+		28 (52)	26 (48)		26 (48)	28 (52)	
PR	-	86	21 (49)	22 (51)	0.829	19 (44)	24 (56)	0.829
	+		27 (47)	23 (54)		20 (47)	23 (53)	
ERα	-	86	9 (82)	2 (18)	0.022	8 (73)	3 (27)	0.06
	+		32 (43)	43 (57)		31 (41)	44 (59)	
ERβ	-	86	8 (100)	0 (0)	0.099	7 (88)	1 (12)	0.247
	+		49 (70)	25 (30)		44 (60)	29 (40)	
ERα/ERβ	low	86	33 (52)	30 (48)	0.148	31 (49)	32 (51)	0.328
	high		8 (35)	15 (65)		8 (35)	15 (65)	
Ki67	low	85	8 (33)	16 (67)	0.145	8 (33)	16 (67)	0.234
	high		31 (51)	30 (49)		29 (48)	32 (53)	

Abbreviations: Lymphovascular space invasion (LVSI), lymph node (LN).

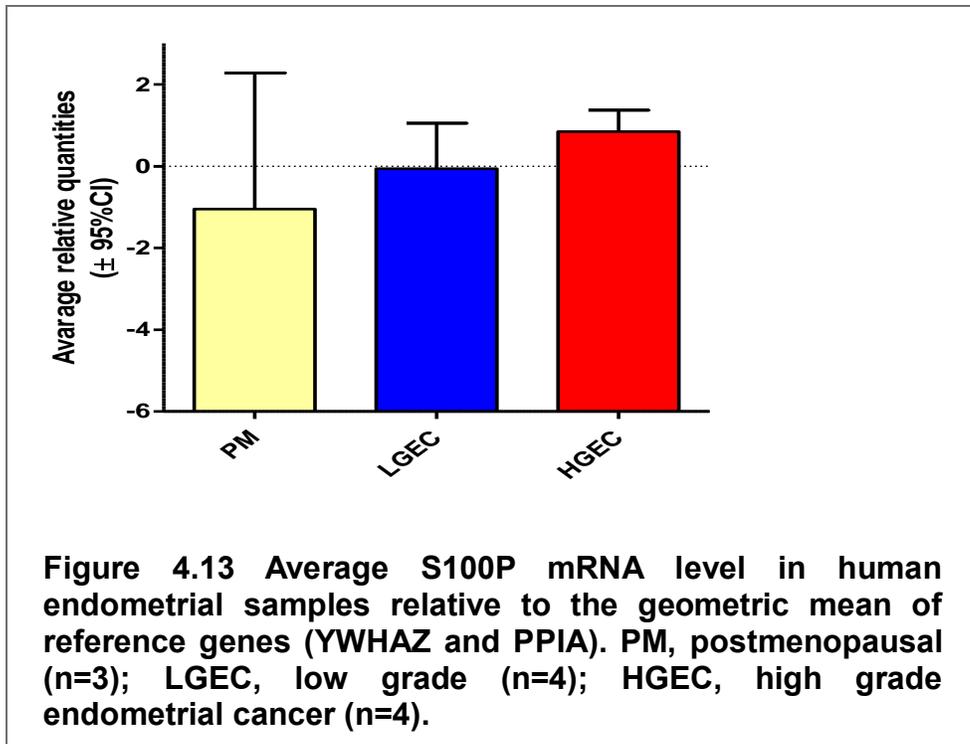
4.4.3.1.6 Survival analysis

The immunoexpression of S100P was examined in the entire EC cohort. As Figure 4.12A and B demonstrate, 12/52 (23%) patients with nuclear S100P+ cancers developed recurrence and 10/51 (20%) deceased within 3 years of their primary surgery compared with 7/49 (14%) and 4/49 (8%) with S100P- tumours respectively, yet the DFS, CSS or OS were not significantly different between the two groups (Figure 4.12A-C). A similar pattern was observed with cytoplasmic S100P expression.



4.4.3.2 The mRNA level of S100P in human endometrial tissue

As Figure 4.13 shows and consistent with the IHC data, S100P mRNA was low in normal PM endometrium. A higher mRNA level was seen in ECs which was more pronounced in HGEC, however, this observation did not show a statistically significant difference (KW, $P=0.0515$).



4.4.4 AGR2

4.4.4.1 IHC results

AGR2 protein was observed in the epithelial cells of normal; pre and postmenopausal endometrium and in the neoplastic endometrium. In all cases, the stromal compartment did not show any immunoreactivity to AGR2 (Figure 4.14). In the epithelial cells, AGR2 was observed exclusively in the cytoplasm.

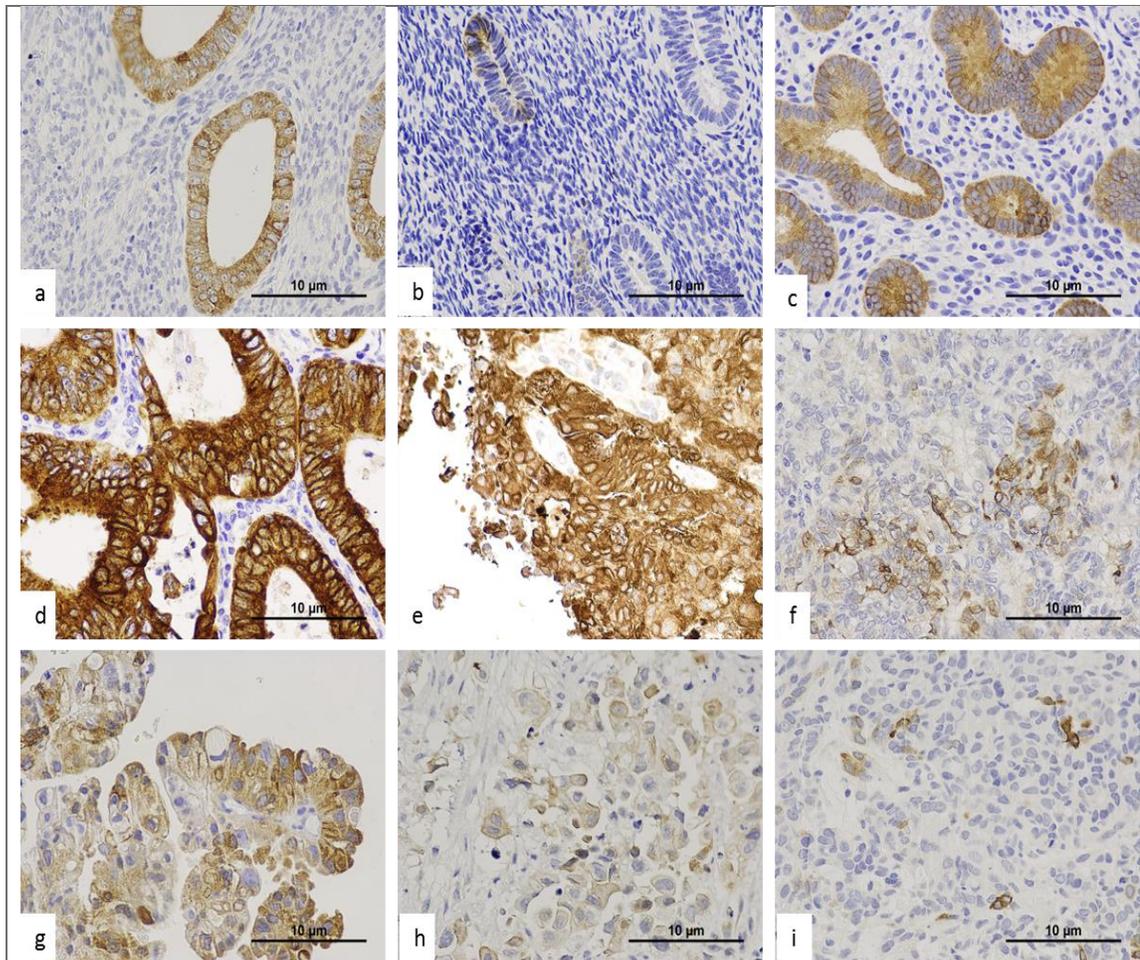
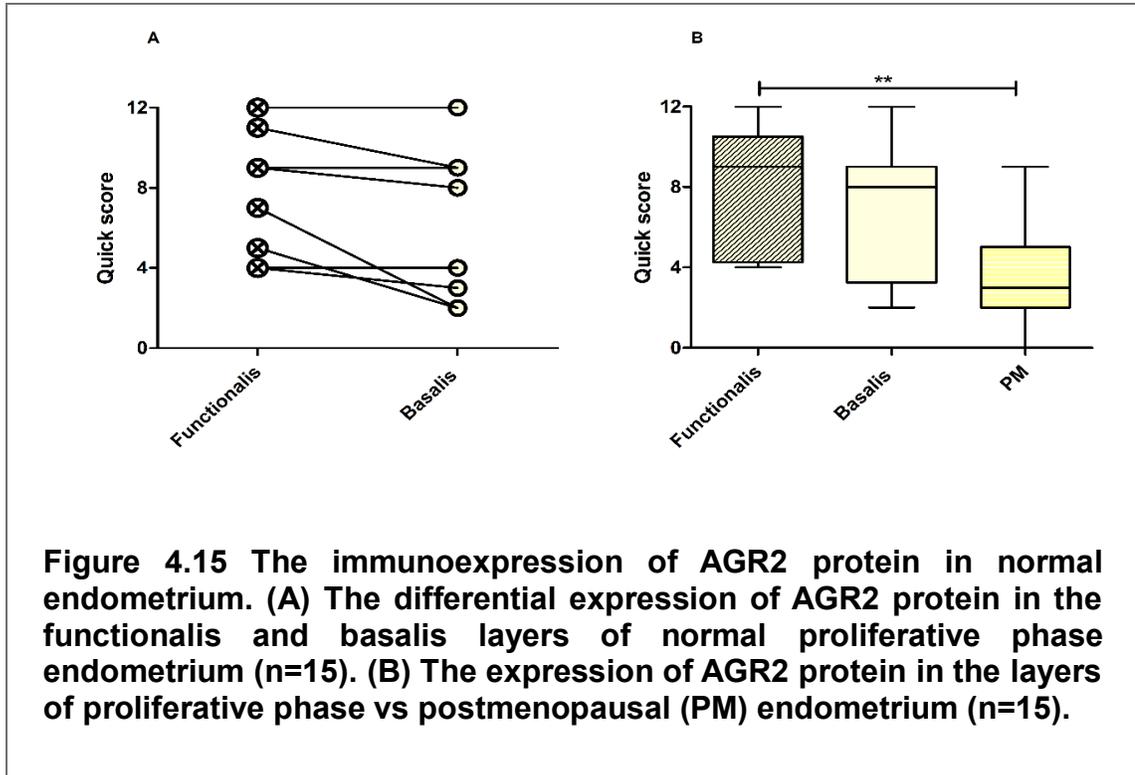


Figure 4.14 Representative microphotographs illustrate the immunoeexpression of AGR2 protein in normal, premalignant and malignant endometrium. AGR2 expression in normal proliferative phase (a), and postmenopausal endometrium (b); hyperplastic endometrium (c); endometrioid endometrial cancer grade1-3 (d-f), serous (g), clear cell (h) and carcinosarcoma (i). Magnification x400.

4.4.4.1.1 Functional layer of normal proliferative phase endometrium expresses higher AGR2.

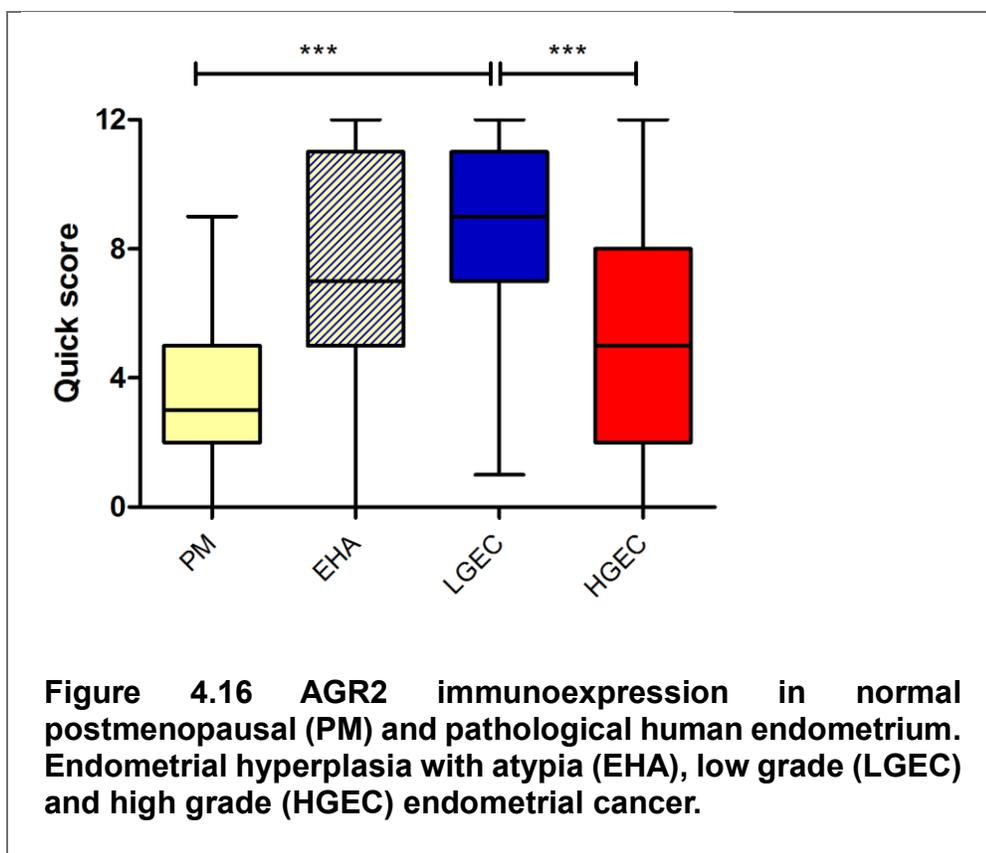
The immunoeexpression of AGR2 in PP glandular epithelia ranged from moderate to strong and was higher in the functionalis layer compared with the matched basalis layer (P= 0.021, Figure 4.15A). There was no significant difference in

AGR2 expression between healthy PP basalis and PM endometrium; however, as expected, the expression of AGR2 in PM endometrium was significantly lower than PP functionalis (P=0.004, Figure 4.15B).



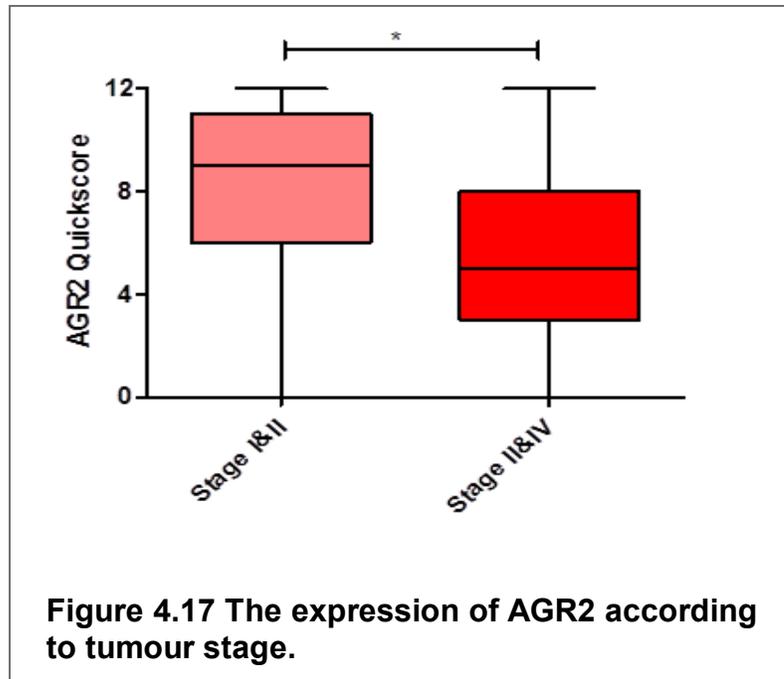
4.4.4.1.2 AGR2 is overexpressed in endometrial hyperplasia with cytological atypia

EHA expressed significantly higher AGR2 (P=0.008) compared with PM (Figure 4.16), while immunoscores of samples without cytological atypia were similar to that of PM. When these results were adjusted for multiple testing, the observed statistical significance in EHA was lost (P=0.116).



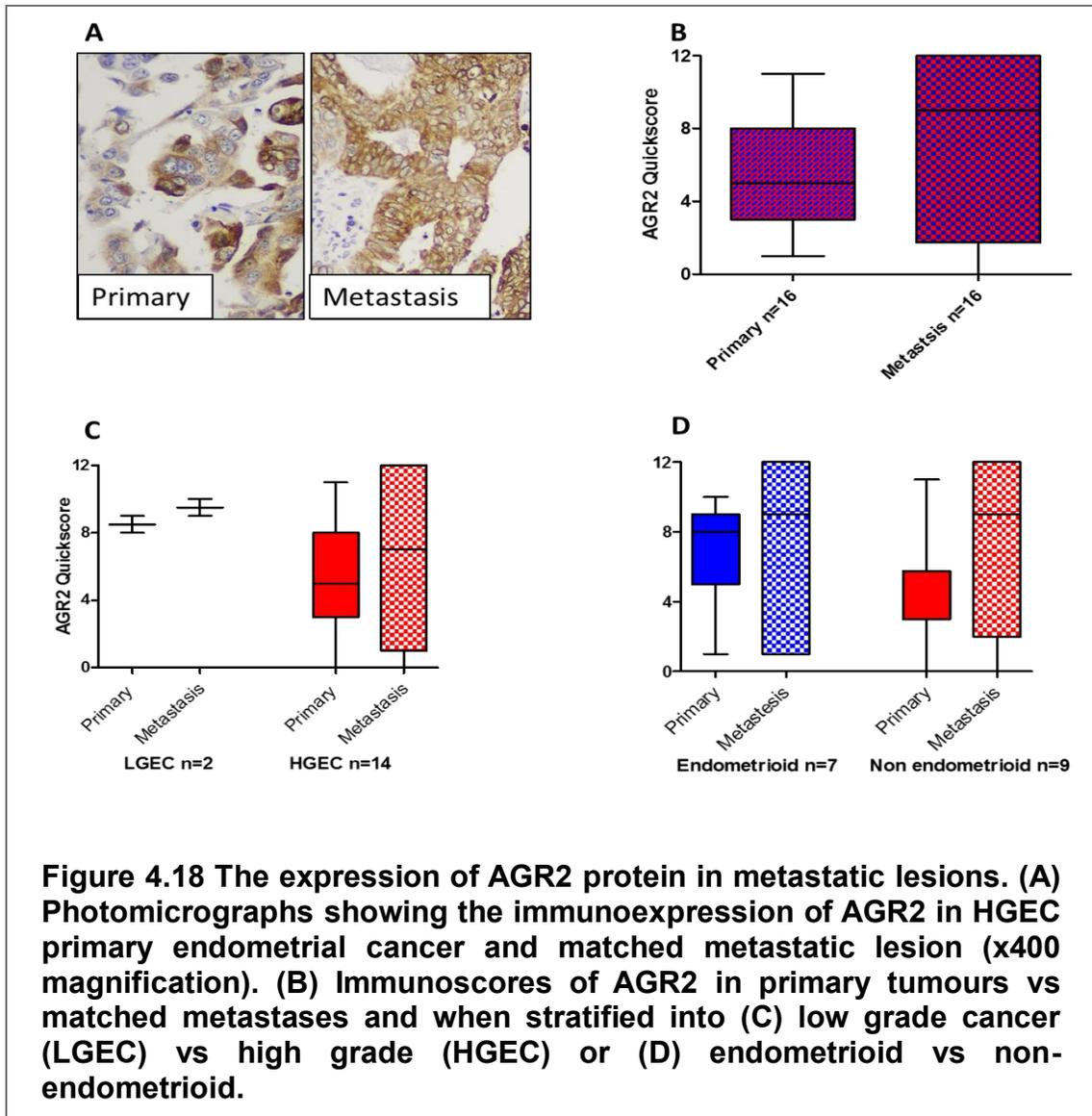
4.4.4.1.3 AGR2 is upregulated in low grade endometrial cancers

As Figure 4.16 shows, AGR2 was significantly upregulated in EC ($P=0.013$) compared with the healthy PM control. When samples were stratified according to tumour grades, LGEC showed the highest AGR2 expression which was statistically significant compared with PM ($P<0.0001$) and PP ($P=0.013$). By contrast, the expression of AGR2 in HGEC significantly less than LGEC ($P<0.0001$) but was not statically different compared with PM. This was also the case within the endometrioid ECs; G3 endometrioid ECs showed a significant reduction in AGR2 immunoscores compared with G1 and G2 ($P=0.016$). Moreover, early stage ECs (I and II) showed a higher AGR2 expression than the advanced stages III and IV ($P=0.012$).



4.4.4.1.4 Metastatic lesions maintain AGR2

AGR2 was expressed in 13/16 (81%) metastatic lesions and although the AGR2 expression appeared to be higher in the metastatic lesions than in matched primary tumours, this difference did not reach statistical significance as shown in Figure 4.18A and B. This was more apparent in the metastatic lesions from HGEC primaries than those from LGEC, particularly in those of nonendometrioid histological type (Figure 4.18B and C). By contrast, cancer cells of lesions metastasised from endometrioid histological type primaries expressed comparable AGR2 scores to those of matched primary tumours Figure 4.18C. None of the differences reached statistical significance.



4.4.4.1.5 The upregulation of AGR2 associates with steroid hormone receptors expression.

ECs were categorised according to AGR2 expression to compare expression in relation to clinicopathological parameters. The expression of AGR2 has not been previously reported in EC; therefore, several cut-off points were applied to identify the best value that could ascertain a group with worst clinicopathological features and worst outcome. Quick scores 2, 3, 4 and 5 were examined for utility. Samples with scores lower than the tested cut-off figure were considered as *low*AGR2 and those with scores equals to or more were considered as *high*AGR2. Out of the

four tested cut-off points, the score of 5 showed the best categorisation, thus considered as the final cut-off figure for this study (Table 4.6).

ECs with *highAGR2* immunoexpression were significantly associated with LGEC ($P<0.0001$) and early stages of cancer ($P=0.003$). No significant association was between *AGR2* and deep myometrial invasion, LVSI, cervical stromal invasion or extra-uterine invasion; However, *highAGR2* immunoexpression was significantly associated with positive AR ($P=0.038$), positive PR ($P=0.043$) and positive ER α , ($P=0.01$). In LGEC (Table 4.7), all cancers that had deep myometrial invasion, cervical stromal invasion and 94% with LVSI expressed *highAGR2*, yet that association did not show statistical significance.

Table 4.6 The association of AGR2 immunoeexpression with clinicopathological factors using four different cut-off points.

AGR2 cut-off variables	total	2			3			4			5							
		-	%	+ %	-	%	+ %	-	%	+ %	-	%	+ %					
Age	<=65	7	(15)	40	(85)	10	(21)	37	(79)	11	(23)	36	(77)	14	(30)	33	(70)	0.401
	>65	12	(23)	41	(77)	15	(28)	38	(72)	18	(34)	35	(66)	21	(40)	32	(60)	
BMI	<30	7	(22)	25	(78)	7	(22)	25	(78)	9	(28)	23	(72)	11	(34)	21	(66)	0.606
	>=30	7	(19)	29	(81)	8	(22)	28	(78)	9	(25)	27	(75)	10	(28)	26	(72)	
Grade	LG	5	(10)	46	(90)	4	(8)	47	(92)	5	(10)	46	(90)	8	(16)	43	(84)	<0.0001
	HG	14	(29)	35	(71)	21	(43)	28	(57)	24	(49)	25	(51)	27	(55)	22	(48)	
Stage	I-II	12	(86)	2	(14)	12	(20)	49	(80)	14	(23)	47	(77)	15	(25)	46	(75)	0.003
	III-IV	7	(19)	30	(81)	13	(35)	24	(65)	15	(41)	22	(59)	20	(54)	17	(46)	
Myometrial invasion	<50	10	(18)	47	(82)	13	(23)	44	(77)	14	(25)	43	(75)	16	(28)	41	(72)	0.085
	≥50	8	(20)	32	(80)	11	(28)	29	(73)	14	(35)	26	(65)	18	(45)	22	(55)	
Cervical invasion	-	14	(20)	56	(80)	18	(26)	52	(74)	21	(30)	49	(70)	23	(33)	47	(67)	0.485
	+	4	(15)	23	(85)	6	(22)	21	(78)	7	(26)	20	(74)	11	(41)	16	(59)	
LVI	-	11	(28)	29	(73)	12	(30)	28	(70)	14	(35)	26	(65)	17	(43)	23	(58)	0.2
	+	7	(12)	51	(88)	12	(21)	46	(79)	14	(24)	44	(76)	17	(29)	41	(71)	
LN	-	0	(0)	8	(100)	4	(50)	4	(50)	4	(50)	4	(50)	5	(63)	3	(36)	0.67
	+	3	(25)	9	(75)	7	(47)	8	(53)	4	(33)	8	(67)	6	(50)	6	(50)	
Extra-uterine invasion	-	4	(13)	27	(87)	10	(32)	21	(68)	11	(35)	20	(65)	14	(45)	17	(55)	0.168
	+	13	(20)	52	(80)	13	(20)	52	(80)	16	(25)	49	(75)	19	(29)	46	(71)	
AR	-	5	(16)	27	(84)	10	(31)	22	(69)	12	(38)	20	(63)	16	(50)	16	(50)	0.038
	+	10	(19)	44	(81)	12	(22)	42	(78)	14	(26)	40	(74)	15	(28)	39	(72)	
PR	-	9	(21)	34	(79)	15	(35)	28	(65)	17	(40)	26	(60)	20	(47)	23	(53)	0.043
	+	6	(14)	37	(86)	7	(16)	36	(84)	9	(21)	34	(79)	11	(26)	32	(74)	
ARPR-	-	11	(18)	51	(82)	14	(32)	48	(77)	16	(26)	46	(74)	18	(29)	44	(71)	0.029
	+	4	(17)	20	(83)	8	(33)	16	(67)	10	(42)	14	(58)	13	(54)	11	(46)	
ERα	-	6	(55)	5	(45)	7	(64)	4	(36)	7	(64)	4	(36)	8	(73)	3	(27)	0.01
	+	9	(12)	66	(88)	15	(20)	60	(80)	19	(25)	56	(75)	23	(31)	52	(69)	
ERβ	-	0	(0)	1	(100)	1	(100)	0	(0)	1	(100)	0	(0)	1	(100)	0	(0)	0.36
	+	15	(18)	70	(82)	21	(25)	64	(75)	25	(29)	60	(71)	30	(35)	55	(65)	
ERα/ERβ	Low	13	(21)	50	(79)	16	(25)	47	(75)	19	(29)	44	(71)	23	(37)	40	(63)	0.883
	high	2	(9)	21	(91)	6	(26)	17	(74)	7	(30)	16	(70)	8	(35)	15	(65)	
Ki67	Low	2	(8)	22	(92)	2	(8)	22	(92)	3	(13)	21	(88)	3	(13)	21	(88)	0.037
	high	14	(23)	47	(77)	19	(31)	42	(69)	22	(36)	39	(64)	27	(44)	34	(56)	0.011

Table 4.7 The association of AGR2 immunorexpression with the clinicopathological parameters in low grade endometrial cancer group (quick score 5 is the cut-off point).

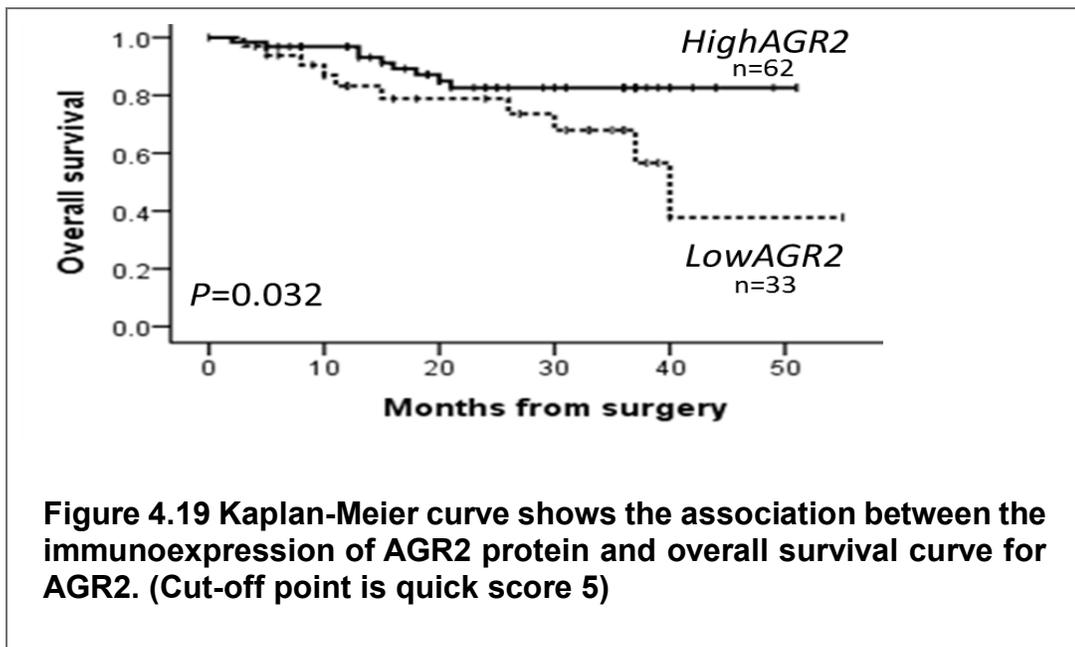
variables	total	AGR2				P
		<5	%	≥5	%	
Age	≤65	48	2 (7)	26 (93)	1	
	>65		1 (5)	19 (95)		
BMI	<30	39	1 (6)	16 (94)	1	
	≥30		2 (9)	20 (91)		
Stage	I-II	49	5 (12)	35 (88)	0.151	
	III-IV		3 (33)	6 (67)		
Myometrial invasion	<50	46	3 (9)	30 (91)	0.548	
	≥ 50		0 (0)	13 (100)		
Cervical invasion	-	46	3 (8)	37 (92)	1	
	+		0 (0)	6 (100)		
LVI	-	47	1 (8)	12 (92)	1	
	+		2 (6)	32 (94)		
Extra-uterine invasion	-	47	0 (0)	9 (100)	0.61	
	+		3 (8)	35 (92)		
AR	-	38	0 (0)	8 (100)	0.58	
	+		3 (10)	27 (90)		
PR	-	38	0 (0)	8 (100)	0.587	
	+		3 (10)	27 (90)		
ARPR-	-	38	3 (9)	32 (91)	1	
	+		0 (0)	3 (100)		
ERα	-	38	0 (0)	2 (100)	1	
	+		3 (8)	33 (92)		
ERβ	-	38	3 (10)	28 (90)	0.614	
	+		0 (0)	7 (100)		
ERα/ERβ	Low	38	2 (9)	20 (91)	0.748	
	high		1 (6)	15 (94)		

Abbreviations: Lymphovascular space invasion (LVSI).

4.4.4.1.6 Survival analysis

*High*AGR2 immunoexpression was significantly present in the cancers of patients with longer OS ($P=0.032$, Figure 4.19), but there was no association with the DFS or CSS. Steroid hormone regulation of AGR2 in the breast and the prostate cancers is well established; therefore, we stratified the samples according to the steroid receptor status and compared the impact of AGR2 on the OS (Figure 4.20). Interestingly, *high*AGR2 was associated with longer OS when AR and/or PR were positive ($P=0.007$) and ER α /ER β was low ($P=0.001$) suggesting a regulatory role of AR and ER in function of AGR2 in human endometrium, although AGR2 expression in steroid receptor positive vs steroid receptor negative strata did not reach statistical significance.

When LGEC, which expressed the highest AGR2 immunoexpression, was considered, women with recurrent disease or those who died were limited to 5 out of 50. This low number of events precluded our ability to assess the prognostic value of AGR2 in that particular group; however, interestingly, *high*AGR2 immunoexpression was observed in 4/5 (80%) of this group.



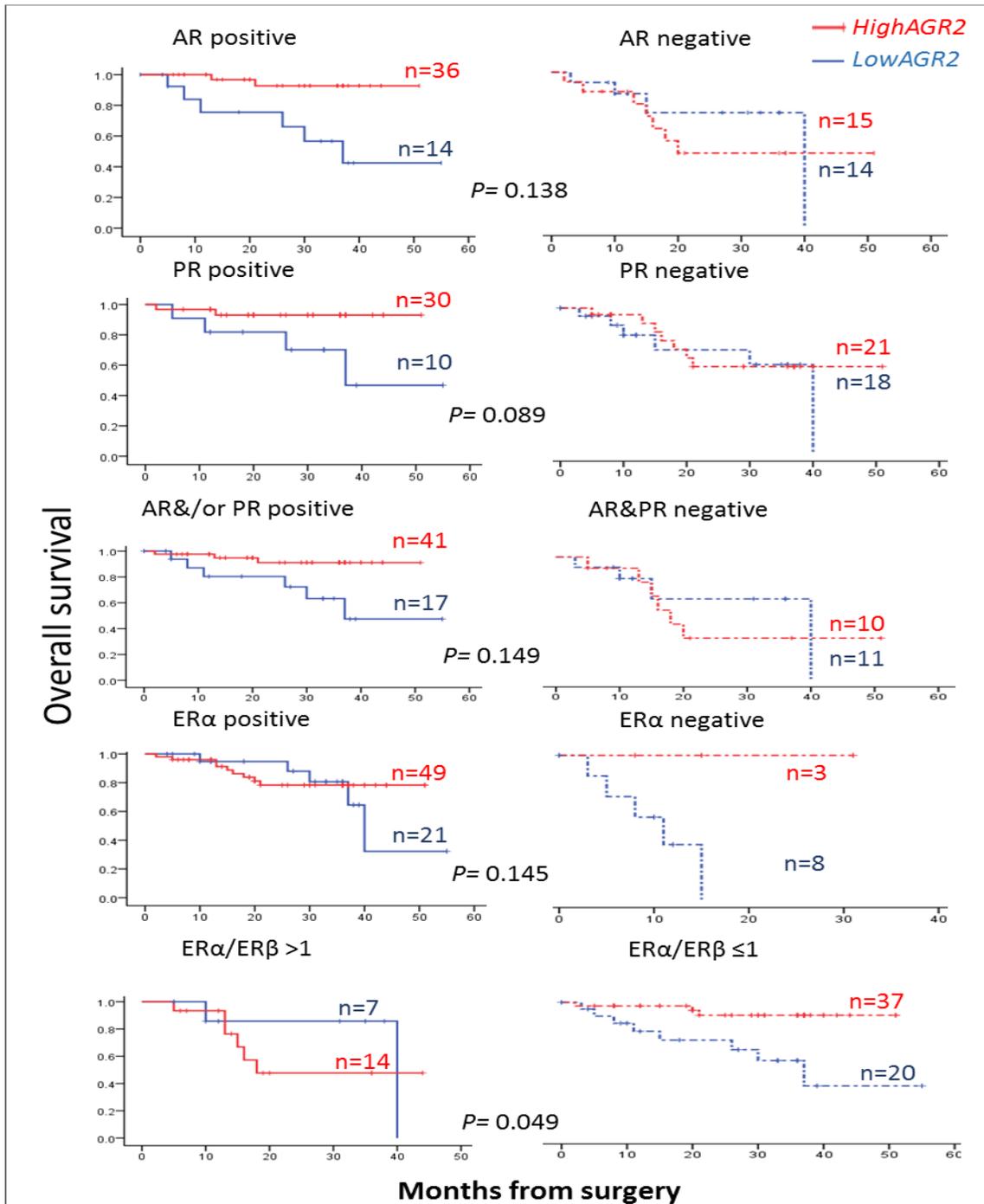
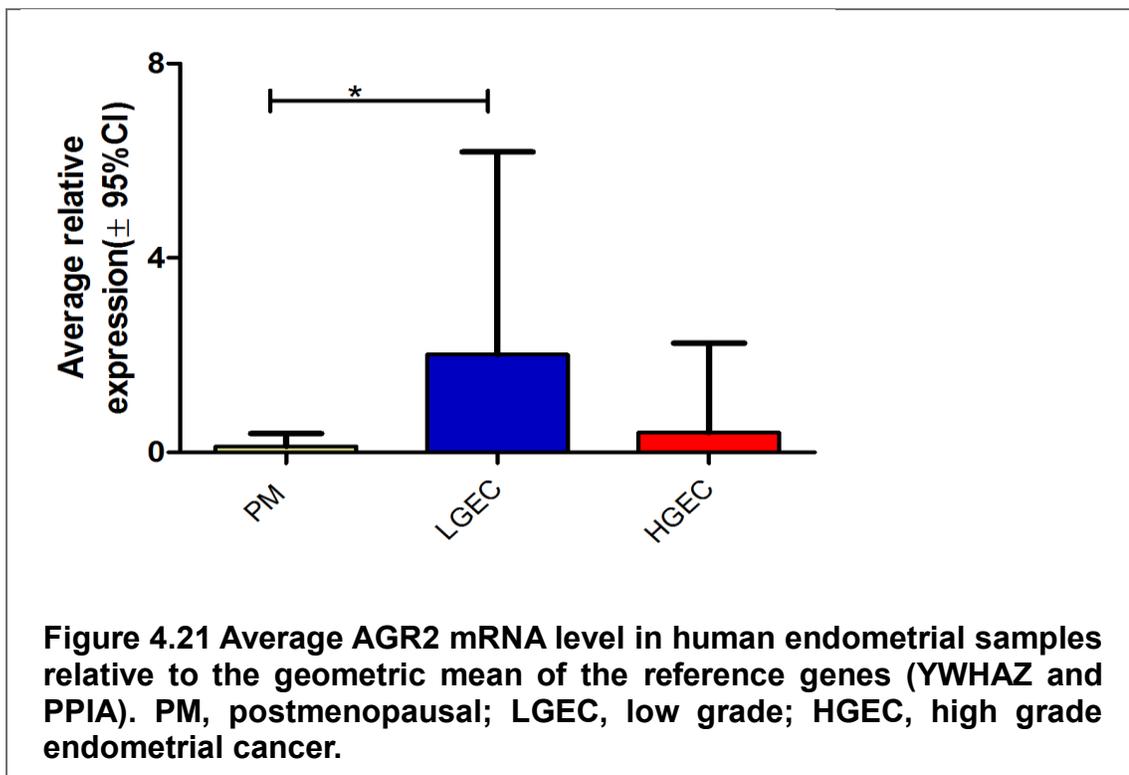


Figure 4.20 Kaplan-Meier curves show the difference between the overall survival of patients with *highAGR2*/ *lowAGR2* in the presence and absence of steroid receptors.

4.4.4.2 The mRNA levels of AGR2 mirror the protein expression

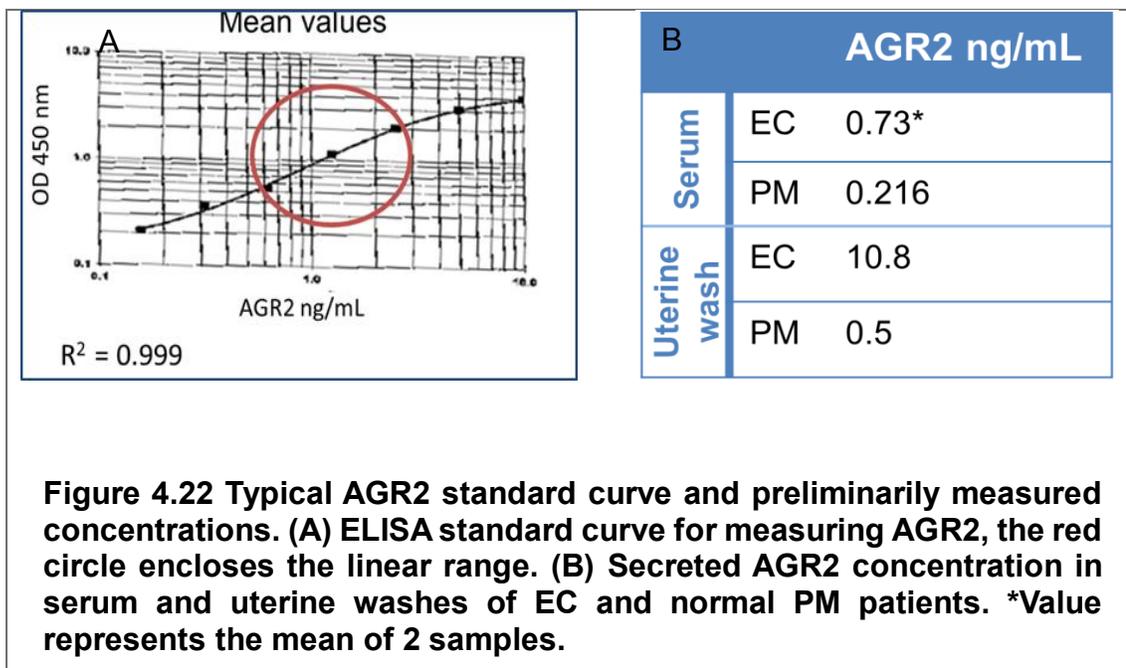
The level of AGR2 mRNA in human endometrial tissue was comparable with the protein immunoeexpression (Figure 4.21). AGR2 transcript level was higher in LGEC compared with PM (P=0.015) and HGEC (P=0.072).



4.4.4.3 ELISA

The correlation coefficient for the standard curve was 0.999, while the linear range of the curve was limited to 0.313-1.257ng/mL as shown in Figure 4.22A; although kit manufacturer indicates that the lower detecting level is 0.156 ng/mL. Although both serum and uterine washes from normal PM and EC individuals showed AGR2; unfortunately, the results were non-reproducible with an unpredicted dose response linearity observed with the kit. Intra-assay variation was determined by repeated (n=3) measurements of high AGR2 samples and confirmed to be 15%. This figure is far beyond the upper limit of the acceptable range of less than 10%. Inter-assay variation was assessed at high, intermediate and low concentration in

three repeated assays producing a very high (accepted variation usually <15%), unacceptable, overall mean coefficient of variation of 26.76 (range; 43.65% for 0.206 ng/mL and 15.5 % for 0.847 ng/mL).



Mean absorbance of 0.156 ng/mL standard was not significantly greater than blank (0.6283 ± 0.24 compared with 0.568 ± 0.21 ; values are means \pm S.D. $n = 2$; $P = 0.860$) indicating that the lower range provided by the manufacturer is invalid. The sensitivity of the kit was tested by spiking 1 pg/ μ l recombinant human AGR2 protein and the standard into a serum sample which showed a recovery of only 12% and 47% respectively. Furthermore, serial dilutions of a spiked serum sample with high AGR2 expression (neat to 1:4) failed to demonstrate a linear dose response relationship. Specificity of the kit for AGR2 was tested by spiking 1pg/ μ l rhAGR3 into a serum sample. No cross reactivity has been observed. Taking all validation tests together, accurate interpolation from the standard curve cannot be expected over a broad concentration range; however, preliminary data from this experiment suggest that local and circulatory level of secretory AGR2 might be higher in women with EC compared with normal post-menopausal women (Figure 4.22B).

4.5 Discussion

This study provides, for the first time, a comprehensive description of MIPs immunoexpression in different histological types of human EC and shows the changes in their expression profile compared to the normal pre and postmenopausal endometrium and EH. It also delineates the utility of these proteins as indicators of tumour progression and mortality. In this section, the results of each protein are first discussed individually then concludes with a brief discussion about the value that a panel of 3 MIPs can further add to identify patients at higher risk of EC progression.

4.5.1 S100A4

Previous studies have established that the upregulation of S100A4 is an inducer of metastasis (Bryony H Lloyd et al., 1998) and an indicator of poor prognosis in EC (Chong et al., 2014). S100A4 has a cell type specific distribution which may contribute to different functions. In normal endometrium, this protein was predominantly expressed in the stromal compartment of PP endometrium consistent with previous research (Xie et al., 2007). We have shown that stromal S100A4 expression was upregulated in normal PM endometrium. Atrophy is the basic morphological picture of human endometrium after menopause. Moderate fibrosis has been described in the atrophic endometrium (Noci et al., 1996). Okada and colleagues (1997) have shown that S100A4 protein is an important early event in the pathway towards EMT and its expression is required for epithelial cells to acquire a fibroblastic phenotype. Our results showed that in normal endometrium, epithelial S100A4 was only expressed after the menopause suggesting that fibrotic changes in PM endometrium can be a consequence of the EMT process regulated by S100A4. This fibrotic change in PM endometrium could be part of normal aging process as it has been previously described in animals (Han et al., 1989) or it may be associated with parity (Noci et al., 1996)

In endometrial cancer, S100A4 was significantly expressed in neoplastic epithelial cells consistent with previous reports (Xie et al., 2007; Chong et al., 2014). We have shown that S100A4 was upregulated in the nuclei of cancer cells and this was strongly correlated with the cytoplasmic expression. Epigenetic and genetic mechanisms have been associated with overexpression of S100A4 protein in EC. Xie and colleagues (2007) have detected hypomethylation of S100A4 mRNA and protein in grade3 endometrioid ECs and TCGA somatic copy number module identified overexpression of S100A4 gene in cluster 2 and 3 which constitute mainly endometrioid EC (Kandoth et al., 2013). *In vitro*, endometrial cancer cells migration and invasion induced by TGF- β 1 was hindered by S100A4 silencing (Xie et al., 2010). TGF- β 1 is a pivotal component of EMT pathway. We have shown that increased S100A4 expression in EC cells was accompanied by a persistent stromal expression which was significantly higher than that of the glandular epithelium. This may suggest an enhanced EMT process, however, the expression of S100A4 in cancer associated stroma was mainly observed in infiltrating leukocytes and macrophages, unlike the PM stroma where S100A4 was predominantly expressed by the fibroblasts. It has been shown recently that the S100A4 protein alters T-cell lineage differentiation shifting T-cells towards the pro-tumorigenic phenotype (Grum-Schwensen et al., 2015). Intriguingly, we have shown that stromal expression was strikingly higher at the invading edge of the tumour, particularly in cancer associated lymphocytes.

In our cohort, epithelial nuclear S100A4 was not significantly different in the high grade and advanced tumours, when compared with the low grade or early cancers as previously reported (Xie et al., 2007); this is likely to be a result of the methodological variance. Xie et al conflicting finding is based on the crude S100A4 transcript levels of both stromal and the epithelial compartments and we have shown that there was a poor correlation between nuclear S100A4 expression and mRNA levels in human EC samples yet the overall mRNA level was the highest in HGEC.

The prognostic significance of S100A4 in EC has been previously reported by a Korean group (Chong et al., 2014). They indicated that S100A4+ was associated with high EC grades, advanced stages and LN metastasis. A major limitation of this study was the use of a nonstandard categorisation of prognostic parameters; for example, they considered grade 2 and 3 endometrioid cancers as high grades and compiled stage II, III and IV together as an advanced stage. In our cohort, when standard categorisation was applied, only cases with deep myometrial invasions showed significant expression of S100A4. No association was observed with LN metastasis because LN status was available for only 8 cases as LN sparing is the standard surgical management for low risk EC patients in our centre. It has been previously reported that the upregulation of S100A4 in epithelial cells was associated with significant reduction in the DFS and OS of Korean EC patients (Chong et al., 2014). We have shown that the high nuclear S100A4 was not only associated with reduced OS but also CSS. DFS in our cohort was not affected by S100A4 expression because we were stringent in the definition of DFS. We censored all the cases which died for causes other than EC; when included, results similar to Chong et al. were obtained.

S100A4 was observed in metastatic EC cells and associated stroma. It is not surprising for metastatic cancer cells to express a comparable immunoscore to those of matched primary as S100A4 function in primary EC is expected to be required in metastatic cells, but it was interesting to see S100A4 cancer associated stroma in the secondary sites. Whether these S100A4+ stromal cells were activated local stromal cells, recruited from haematopoietic progenitors (Gupta and Massagu, 2006) or resulted from epithelial malignant cells transformation (Yao et al., 2011) require further investigation. Notably, metastatic lesions isolated from abdominal LN and omentum tend to show higher S100A4 immunoexpression than those from parametria and ovaries which may suggest a potential association with distant metastasis. This can be further studied in a larger sample.

S100A4 expression in normal reproductive endometrium does not show cyclical changes in response to hormone (Hapangama et al., 2012). Our results, consistent with (Xie et al. 2007), showed that there was no correlation between S100A4 and steroid receptor expression in endometrial cancer. By contrast, (Chong et al., 2014) has reported an inverse correlation between S100A4 and PR expression. These conflicting results were further addressed in chapter 5 by examining the role of steroid hormones and their receptors in the regulation of S100A4.

Negative immunoreactivity of normal and hyperplastic endometrial epithelial cells confirms the early evidence by (Nikitenko et al., 2000) contributing this protein in tumour progression rather than initiation

4.5.2 S100P

Dysregulation of S100P in different human cancers has been associated with poor patient outcome and therapy failure (Gibadulinova et al., 2011, 2016; Yuan et al., 2013; Zhao et al., 2013). The change in the expression profile of S100P protein in human EC and its association with tumour initiation and progression is not well defined. In normal endometrium, we have shown, consistent with previous reports, that PP endometrial glandular epithelium in the oestrogen dominant milieu express weak S100P immunoreactivity which was limited to the perinuclear cytoplasmic localisation (Tong et al., 2010; Hapangama et al., 2012; Zhang et al., 2012). Conversely, upregulation of S100P was reported in the progesterone dominant mid-secretory phase when endometrial cells undergo considerable structural changes and migration preparing for subsequent implantation (Tong et al., 2010; Zhang et al., 2012). We have also shown that epithelium of PM glands, when progesterone is at a nadir, expressed weak to moderate S100P which was exclusively in the cytoplasm. In vitro, Zhang et al have demonstrated upregulation of S100P in primary endometrial epithelial cells isolated from the proliferative endometrium in response to a high dose of progesterone but not oestrogen

(Zhang et al., 2012). Taking together, these findings may support progesterone regulation of cytoplasmic S100P.

Activation of S100P regulates cell proliferation/survival, cell motility and invasion through intracellular or extracellular signalling pathway (Jiang et al., 2012). The cellular localisation of S100P corresponds to different molecular functions. Cytoplasmic S100P is involved in the degradation of β catenin by binding to CacyBP/SIP, a component of ubiquitin pathway (Filipek, 2002). Canonical Wnt/ β -catenin pathway has shown to be inactive in normal preM and PM. Nguyen et al. (2012) have localised β -catenin to the cell membrane which may suggest the involvement of cytoplasmic S100P in signalling pathways other than Wnt/ β -catenin in normal endometrium.

In EC, downregulation of cytoplasmic S100P was observed. The loss of cytoplasmic S100P was associated unfavourable prognostic factors such as older age, LVSI and cervical stromal invasion but did not affect DFS, CSS, or OS. The canonical Wnt/ β -catenin pathway is active in EC and β -catenin has been localised to the nuclei of endometrial cancer cells (Saegusa et al., 2003). It is tempting to speculate that the downregulation of cytoplasmic S100P reduces β -catenin degradation and increases its bioavailability resulting in a further increase in cell proliferation.

Intriguingly, the loss of cytoplasmic S100P was associated with significant nuclear translocation of S100P in both the LGEC and HGEC samples including the non-endometrioid subtypes compared with PM. The observed nuclear translocation can be a result of structural changes in S100P gene or protein. Genetic profiling of EC (TCGA dataset) has reported a deletion mutation in S100P gene in the cluster 3 and 4 cancers (Kandoth et al. 2013). A truncation in the C-terminus of S100P protein has been recently reported in breast cancer and was associated with a restricted nuclear localisation and a worse outcome (Chung et al., 2015). The exact function S100P plays in the nucleus remains unclear. S100P binding protein (S100PBP) has been shown to bind S100P and co-localise with it in the

nucleus of pancreatic cancer cells (Downen et al., 2005); this nuclear S100P–S100BP complex may be involved in the oncogenic process by cooperation with different cathepsins in organ and disease specific pattern (Lines et al., 2012).

The expression of S100P in human endometrium has been recently reported (Guo et al. 2014), however, examination of the figures presented in that paper reveals a clear misinterpretation of the inflammatory cells infiltrating EC to be S100P+ neoplastic cells which disqualify their results and conclusions.

There was also a differential expression of S100P between different EC histological sub-types, for example, endometrial clear cell carcinomas expressed relatively high levels of S100P whereas scanty S100P expression was observed in the endometrial serous cancers. An opposite pattern has been described for the same histological subtypes of ovarian cancer. Umezaki et al. (2015) reported a low S100P expression in ovarian clear cell whereas serous ovarian cancer was reported to express high S100P (Wang et al., 2015). The differential expression of S100P in ovarian clear cells and serous cancers was associated with different prognostic implications, from being a good prognostic indicator in ovarian clear cell carcinoma and a poor prognostic indicator in ovarian serous carcinoma (Umezaki et al., 2015; Wang et al., 2015). Likewise, we have noticed that when clear cell EC (not serous EC) expressed high nuclear S100P, patients were more likely to develop a progressive disease but the number of samples was insufficient to make a conclusion.

Nuclear S100P expression did not show a significant correlation with any of the unfavourable prognostic parameters examined except ER α and was not able to predict patient's DFS, CSS or OS when all subtypes were analysed together. This is likely to be due to the variety of histology subtypes being included in the survival analysis. S100P may have different roles and may be involved in different pathways in the context of different cancers, and this was particularly evident when serous cancer was considered against the others as previously discussed;

however, unfortunately, the number of samples in our cohort was insufficient to carry out survival analysis for each histological subtype individually.

S100P protein localisation in the metastatic lesions combined normal and primary EC S100P expression profile. S100P was expressed in the nuclei of metastatic EC cells with immunoscores relatively comparable to those of matched primary. Cytoplasmic S100P immunoscores of metastatic cells, however, were significantly higher than matched primary, particularly in nonendometrioid tumours. Metastatic cells require anchoring, adhesion and proliferation/ survival to establish in the secondary tissue. Increased cytoplasmic S100P may support metastatic colonisation via two pathways; the first is by increasing cells proliferation via β -catenin/ Wnt pathway. Activated Wnt pathway was reported in the metastatic cells of some cancers such as colon metastatic EC (Hugh et al., 1999). The second possibility is by facilitating metastatic cells extravasation in the secondary tissue via cytoskeletal regulator ezrin, a binding partner of S100P which has a role in cell differentiation, adhesion and migration (Austermann et al. 2008).

In conclusion, the expression of S100P protein in nuclear localisation may associate with endometrial carcinogenesis. The differential pattern of S100P in EC subtype may associate with different functions. S100P expression did not show prognostic significance. The association of S100P expression with ER α in EC incurs further investigation.

4.5.3 AGR2

AGR2 is a protein expressed in many solid tumour types. Oncogenic properties of this protein are mediated via promoting cell proliferation, cell survival, and metastasis (Liu et al., 2005; Vanderlaag et al., 2010). Results presented in this chapter demonstrated for the first time the differential changes in the expression of AGR2 protein in normal human endometrium and different types of EC. In the normal endometrium, the immunoexpression of AGR2 in PM glandular epithelium was similar to that of basalis layer of PP which known to be less responsive to

ovarian hormones. The hormonally dynamic functionalis layer showed a higher immunoexpression of AGR2 in the glandular epithelium of PP which was significant compared to PM. Our group has previously reported a high expression of AGR2 in the functionalis of normal mid secretory phase glandular epithelium which was higher than PP (Hapangama et al., 2012); however, the expression pattern was different in terms of compartment staining. They have shown that stromal cells in PP were immunoreactive to AGR2. We used a monoclonal commercially available Ab with high sensitivity to AGR2 and specificity (no cross-reactivity with AGR3). When we compared samples stained with the current Ab and that used in the previous study, Immunoreactivity to AGR2 in endometrial stromal cells or endothelial cells was not detected and epithelial expression was slightly weaker. These results suggest a hormonal regulation of AGR2 in normal endometrium which is further supported by the overexpression in hyperplastic endometrium developed after menopause. Moreover, upregulation of AGR2 was significant in LGEC which expressed a relatively high level of steroid receptors compared with HGEC. Both ER and AR have been previously mapped on the promoter of AGR2 and upregulation of AGR2 by oestrogen agonists/antagonist and androgen have been reported (Hrstka et al., 2010; Bu et al., 2013) indicating an intricate interaction between different steroid hormones/receptors and AGR2 in different contexts.

To evaluate the impact of AGR2 immunoexpression on the outcome of EC, survival analysis was done first on the entire cohort which included various histological types of EC with different steroid receptor expression levels. The OS of EC patients was better when cancer immunoscores of AGR2 were high, but that was not maintained in CSS where there was an overlap between the *highAGR2* group and the other group. *highAGR2* was significantly associated with a positive steroid receptor expression and we have shown in chapter 3 that AR, PR as well as ER α were predictors of better OS. It is possible that the increased OS rate may be due to the improved OS conveyed by steroid receptors. Therefore, we further stratified patient survival according to the expression of steroid expression and as expected, we found that in the presence of AR and/or PR and

low ER α / ER β , patients with *highAGR2* had better OS survival, however, a larger sample will be required to come to a firm conclusion in this regard. It is well established that molecular drivers of LGEC are different from those of HGEC (Morice et al., 2015). By examining TCGA data, AGR2 is overexpressed in the worst outcome serous like cluster (Kandoth et al., 2013). Although 94% of serous EC were grouped in this cluster, 12% of all endometrioid samples were also included, and interestingly, 5% of those were LGEC. We have shown that AGR2 expression was significantly downregulated in HGEC. It is tempting to speculate that overexpression of AGR2 is an adverse event in LGEC. LGECs are generally characterised by a very good prognosis with the 5-year survival exceeding 85%, yet 5-15% of the women with LGECs are still prone to relapse (Colombo et al., 2013). Although we are particularly interested in this group, with such low incidence of relapse, it was challenging to confirm the ability of AGR2 to predict progression of LGEC in our cohort. The adverse outcome in 85% of LGEC with *highAGR2* observed in here therefore warrants further study.

The expression of AGR2 in relation to EC progression was then evaluated in the metastatic lesions. Interestingly, not only metastatic lesions from LGEC expressed *highAGR2*, but 57% of those from HGEC group also expressed an increased level of AGR2 compared with the matched primary cancer. These lesions also expressed high levels of AR and ER α . Settlement of metastatic cells in the new microenvironment of the secondary tissue is a stressful event in cellular terms (Pani et al., 2010). The expression of steroid receptors can add a further endocrine stimulated stress by inducing large alterations in of gene transcription (Salmans et al., 2013). The involvement of AGR2 in protein folding and endoplasmic reticulum-assisted degradation (Higa et al., 2011) may, therefore, allow tumour cells to avoid cell death.

Over the past few years, the secreted AGR2 has received a considerable amount of attention as a cancer biomarker. Elevated serum AGR2 has been associated with poor outcome in patients with ovarian, prostate and lung cancers (Edgell et al., 2010; Chung et al., 2012; Kani et al., 2013). Similarly, local upregulation of

secreted AGR2 in the urine of prostatic cancer patients showed a correlation with tumour recurrence (Wayner et al., 2012). Interestingly, elevated levels of locally secreted AGR2 in pancreatic juice was able to identify patients with premalignant pancreatic lesions (Chen et al., 2010). In human endometrial samples, we have shown that AGR2 immunoexpression was elevated in endometrial hyperplasia with cytological atypia compared with the age-matched PM endometrium, and this AGR2 expression was further increased in low grade and early stages of EC. Therefore, the utility of AGR2 as a biomarker for early detection of EC was further investigated. We used a commercially available protein binding assay which has been previously used for AGR2 detection in serum and body fluids (Makawita et al., 2011; Chung et al., 2012; Kani et al., 2013). The kit failed the quality control tests and its precision was not confirmed. Considerable modification to the manufacturer's protocol has been made by other studies suggesting that they also encountered similar issues. Although we discontinued this experiment, we have shown that AGR2 is secreted by endometrial cancer cells; and the levels were higher in uterine wash compared to that of the serum.

Taking the above presented information together, the upregulation of AGR2 is an early event in endometrial carcinogenesis and associates with well differentiated tumours but may also contribute to tumour progression and metastasis. Furthermore, the expression of AGR2 in EC is likely to be under the regulation of ovarian hormones.

4.5.4 Impact of a panel of MIPs on clinicopathological parameters and survival

One of our aims was to investigate the synergistic effect of a panel of three MIPs over the individual protein prediction of patient's survival. Primary evaluation of the prognostic value of the MIPs individually indicated that the OS and CSS of EC patients expressing S100A4 protein in the tumour were shorter than those with S100A4-, whereas S100P did not show association with patient survival. Cox multivariate analysis did not identify S100A4 to have an independent prognostic

value. AGR2, on the other hand, has a different expression profile and if there is a prognostic value in AGR2, at least in the LGECs which are going to recur, that possibility requires further investigation in a much larger cohort of LGECs than presented here. Thus, my data does not suggest an added value for using a panel of the 3 proteins together.

Chapter Five

**Hormonal Modulation of
Steroid Receptors and MIPs**

5.1 Introduction

The importance of sex steroid hormones in the development of endometrioid endometrial cancer is well established. The majority of low grade endometrial cancers (LGECs) express ER, PR, and AR, similar to the healthy endometrium (Kamal et al. 2016). Circulating androgens and oestrogens are significantly upregulated in endometrial cancer (EC) patients compared with normal postmenopausal (PM) women with a concomitant reduction in the sex hormone binding globulin, SHBG, suggesting an increased availability of these hormones to the EC tissue (Allen et al., 2008). Several lines of evidence also exist demonstrating the importance of the local metabolism of hormone, which dictates the hormone milieu of the peripheral tissues, including the endometrium (Huhtinen et al., 2012; Sinreih et al., 2013).

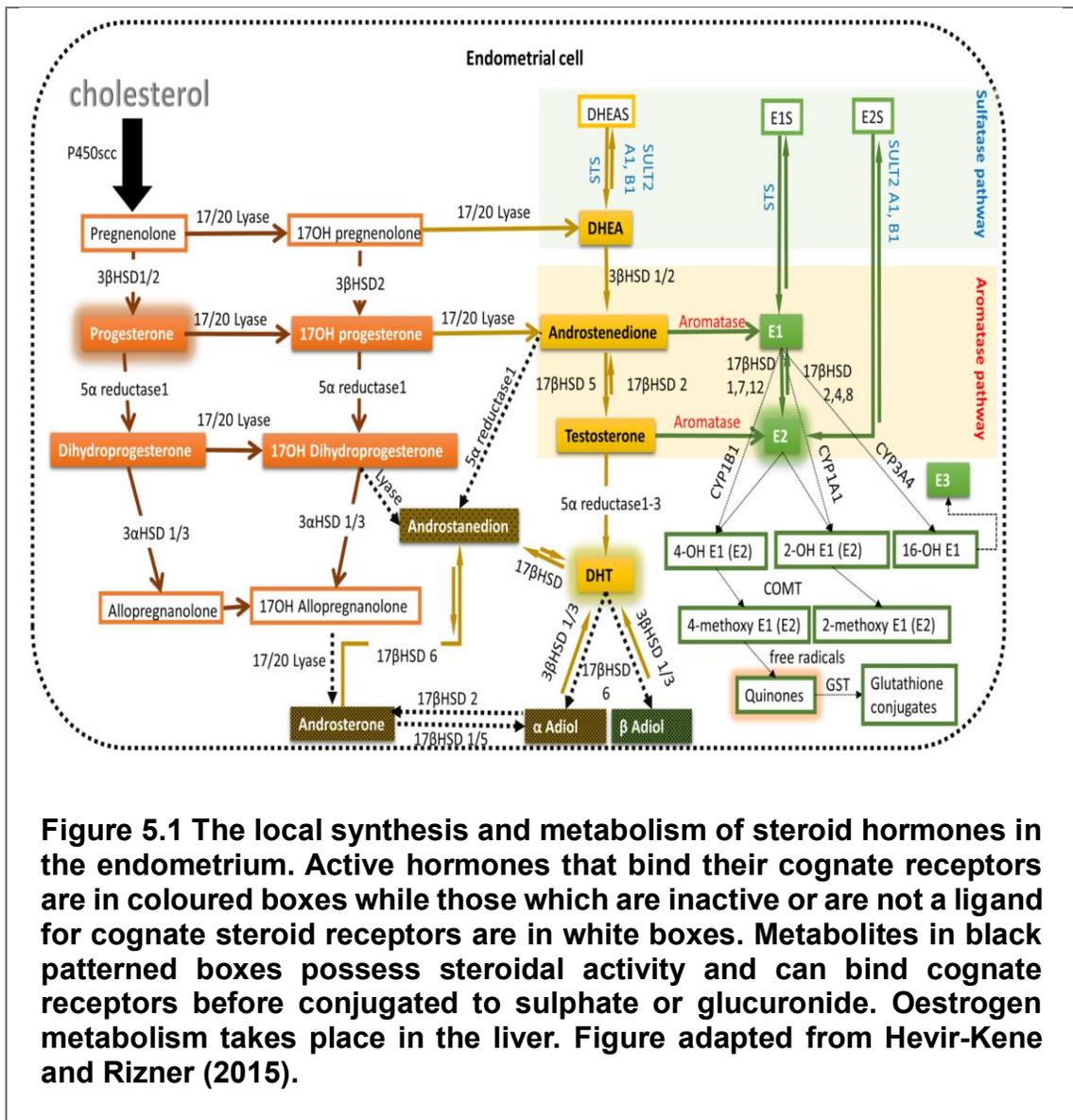
Oestrogens are pivotal hormones regulating the growth of EC through binding with ER (Kamal et al., 2016a). Although ERs are downregulated in high grade endometrial cancer (HGEC), a weak to moderate ER β expression with/without ER α was always seen in at least 10% of the malignant cells (Kamal et al. 2016). Local E2 production can occur via two pathways as shown in Figure 5.1: the aromatase pathway, in which oestradiol (E2) is formed from Dehydroepiandrosterone (DHEA), DHEA-sulphate, androstenedione and testosterone; and the sulfatase pathway, in which E2 is derived from circulating oestrone sulphate (Hevir-Kene and Rizner, 2015).

Progesterone antagonises the function of oestrogen and alters (down-regulates) the expression of steroid receptors (Lane et al., 1988; Burton et al., 2003). A variety of progestagenic agents have been employed in the treatment of recurrent and metastatic EC for a long time, although the maximum tumour repressive response achieved by them has not exceeded 20% (Thigpen et al., 1999). Interestingly, a regimen of alternating the synthetic progestogen, megestrol acetate with oestrogen receptor modulator, tamoxifen resulted in achieving a prolonged and complete response in up to 33% of the patients (Fiorica et al.,

2004). Almost all available synthetic progestins are derivatives of C19 steroids, and therefore bind with high affinity to PR but also have a significant affinity to AR where they induce androgenic gene transcription (Bentel et al., 1999). In chapter 3, I have already demonstrated that PR, positively correlated with AR and that AR is a positive prognostic indicator similar to PR and its loss is associated with a shorter disease-free survival (Kamal, Bulmer, et al., 2016).

Androgens are generally regarded as the male hormone, due to the well-established critical role they play in the physiological and pathological development of male genital organs, and the involvement of the androgen pathway in prostate cancer. However, its participation in hormonally regulated malignancies in women, has not been fully elucidated, but has been of increased interest to endometrial cancer researchers recently (Rižner and Penning, 2014; Tanaka et al., 2015; Simitsidellis et al., 2016).

The role of androgen in endometrial cells, particularly in relevant functions/pathways in the context of cancer, such as proliferation and metastasis is even less well characterised. Testosterone, dihydrotestosterone (DHT) and to a lesser extent androstenedione and DHEA, are all thought to produce the biological androgenic functions through their associations with AR (Henley et al., 2005). Tuckerman and colleagues have shown that androstenedione can inhibit cell proliferation via AR in primary endometrial epithelial cells isolated from normal premenopausal endometrial biopsies, yet they failed to replicate this inhibitory effect with testosterone or DHT (Tuckerman et al., 2000). DHT is a naturally occurring non-aromatised androgen that circulates in low concentrations in serum with higher affinity to AR (Strauss, 2009). In peripheral, Figure 5.1, tissue such as in the endometrium, DHT is produced from testosterone by the enzyme 5 α -reductase (Ito et al., 2002). Ito and colleagues have shown that 5 α -reductase-1 was overexpressed in the endometrioid ECs and associated with an increased tissue/ serum ratio of DHT and a better patient outcome (Tanaka et al., 2015).



This indicates that an increased local production of DHT and possibly a subsequent activation of the androgenic pathway is associated with a better patient outcome. *In vitro*, the effect of DHT was previously investigated in MFE296 cells which were derived from a moderately differentiated, stage I endometrioid EC (Hackenberg et al., 1994). The proliferation of the MFE296 cells, which doesn't express any of the steroid receptors except AR, was inhibited after approximately 24h of treatment (Hackenberg et al., 1994). This observation suggests a potential therapeutic role for the activated androgenic pathway; however, human endometrial cancers express a range of steroid receptors each at altered levels

in the different EC subtypes. However, ER β was consistently expressed in ECs, including in 89% of our HGEC cohort (chapter 3). Therefore, it is necessary to employ an endometrial cancer cell line for *in vitro* experiments which expresses all four steroid receptors to show the effect of DHT on endometrial cancer cell proliferation and it also allows the examination of the effect of androgens on the steroid receptors expression. We chose to examine the effect of DHT on genes coding for sex steroid receptors, due to their likely subsequent influence on modulating the final effect of all hormones. Although there is (limited) evidence regarding the effects of androgens on endometrial AR expression (Lovely et al., 2000; Apparao et al., 2002), the current understanding of the androgenic effect on the expression of the other sex steroid receptor genes in the endometrium is yet to be fully understood.

Furthermore, in line with the general theme of this thesis, the effect of androgen on the endometrial expression of MIPs genes was also examined. A hormonal regulation of MIPs has also been suggested in some malignancies; for example, oestrogens appear to regulate AGR2 in the breast, prostate, and in ovarian cancer (Barraclough et al., 2009; Verma et al., 2012; Bu et al., 2013; Salmans et al., 2013); and our own data highlighted a significant positive correlation between AGR2 expression with positive ER α , PR and AR receptor expression (Chapter 4). The hormonal regulation of S100 proteins particular to EC is less well defined with conflicting reports of an association between S100A4 with ER and PR expression in EC (Xie et al., 2007; Chong et al., 2014) which may suggest oestrogen regulation; and to our knowledge, there is no information in the literature that explores the hormonal regulation of S100P in EC to date. Upregulation of S100P in the secretory endometrium has been previously reported and that suggests a possible hormonal regulation. Progesterone was shown to induce S100P in premenopausal primary endometrial cells (Zhang et al., 2012); however, we have shown that PM and EC cells expressed higher S100P in the absence of progesterone which may indicate the involvement of the other steroid hormones.

Due the aforementioned contradictions in the current literature regarding the effects of androgens in the human endometrium, the data included in this chapter were obtained from studies carried out to answer the following research questions;

5.2 Research questions

1. How would the expression profile of steroid receptors and MIPs differ in 4 established endometrial cancer cell lines and which cell line could present a suitable model for studying hormone regulation of steroid receptors and MIPs?
2. Can the maximum effects of the potent androgen, DHT, modulate the expression of the steroid receptors when assessed in the appropriate cell line?
3. When all four steroid receptors are present (as in the endometrial epithelial cell line), can DHT inhibit cell proliferation?
4. Does the intact endometrial 3D architecture affect the modulatory effect of the sex steroids on protein expression (steroid receptors, AGR2), and cell proliferation (Ki67) at the protein level?
5. What are the effects of postmenopausal sex steroid hormones on the endometrial expression of MIPs?

5.3 Methods

5.3.1 Human tissue

Nine endometrial tissue biopsies, 6 pipelles and 3 full thickness, were obtained from women at day 10-20 of their regular menstrual cycles, mean age 28.7 (25 – 35) years, at the time of elective surgery for benign, non-endometrial gynaecological conditions (e.g. female sterilisation) with no clinical (symptomatic or surgical) evidence of endometriosis at LWH. None of these women had taken oral contraceptives or other hormonal medications for at least 3 months before the biopsies were taken. Samples were transported in 10mL DMEM. This time of the cycle was selected because the cells obtained in this phase maintain a high degree of proliferation capacity

5.3.2 Culture system

5.3.2.1 Cell lines

Ishikawa (ISK) cancer cell line and human endometrial stromal cell line (hESC) were grown and maintained as previously described in chapter 2.5.1.1 and 2.5.1.2 respectively. The cells were prepared for steroid treatment by preconditioning in DMEM/F12 (phenol red-free, Life Technologies, UK) supplemented with penicillin/streptomycin antibiotic and 2% CS-FBS for 48 hours prior to the experiment. Cells pellets of HEC1A, RL95-2 and MFE280 were provided by CP for characterisation for steroid receptors and MIPs gene expression.

5.3.2.2 Primary cells

Stromal cells were isolated from 6 endometrial biopsies and maintained as described in chapter 2.5.1.3. These cells were also prepared for steroid treatment as described above.

5.3.2.3 Co-culture

Co-culturing ISK cells with hESC was chosen to permit epithelial- stromal cross talk as previously described in chapter 2.5.2. Briefly, ISK cells were seeded in trans-well inserts with 0.4 mm pores at a density of 3×10^5 cell/insert and cultured in DMEM/F12 with 2% CS-FBS for 48h. hESC or primary stromal cells were also simultaneously seeded in 6 well plates at a density of $\sim 1.5 \times 10^5$ and cultured in DMEM/F12 with 2% CS-FBS. After 48h, culture media in the base and in inserts was replaced with 2.6 and 1.5mL DMEM/F12 with 2% CS FBS \pm treatments respectively. Inserts were subsequently placed on the top of the above mentioned bases as shown in Figure 2.1 and the resulting ISK/stromal co-cultures were incubated for 1-3 days. Cells were then harvested using trypsin at the indicated time points, pelleted and stored for PCR studies at -80°C .

5.3.2.4 Explants

Endometrial explants were prepared as previously detailed in chapter 2.5.3. Briefly, the endometrial biopsies were separated from the medium and transferred to a Petri dish, rinsed in PBS to clear the blood and mucus. Subsequently, the tissue was mechanically dissected into pieces of 1–2 mm³ and pre-incubated in DMEM/ F-12 medium with 2% CS-FBS for 30 minutes until hormonal treatment was ready. During the hormone treatment experiments, the explants (3–5 pieces) were placed in a 6-well plate and cultured in 3 mL of fresh 2% CS FBS medium with or without treatment for 24h. Explant cultures were subsequently fixed in 10% NBF for 24h before processing.

5.3.3 Hormone treatment

The steroid hormones were added from 1000-fold concentrated stocks made in absolute methanol to the desired concentration of DHT (final concentration; 1 μM –1 pM) and E2 (final concentration; 1–0.001 μM) individually or in combination and with ER antagonist, fulvestrant (ICI 182,780, final concentration; 1 μM) or AR antagonist, bicalutamide (CDX, final concentration; 1 μM). The duration of

exposure to the hormone or appropriate vehicle in each experiment is detailed in the flow chart Figure 5.2.

5.3.4 Cell proliferation

ISK cells preconditioned with 2% CS-FBS were labelled with CFSE and treated with assigned hormone/antagonist combination as described in chapter 2.6.1 and illustrated in Figure 5.2. Flow cytometric analysis was conducted at the Department of Children's Health/ Alder Hey Hospital, Liverpool as detailed in chapter 2.6.2. The cells were re-suspended in 3mL culturing media in preparation for the FACS analysis. 1mL of single cell suspensions from each respective treatment condition employed was transferred to FACS tube (12 x 75mm tubes) and placed on ice in a dark place during the preparation/ setting up of the FACS machine.

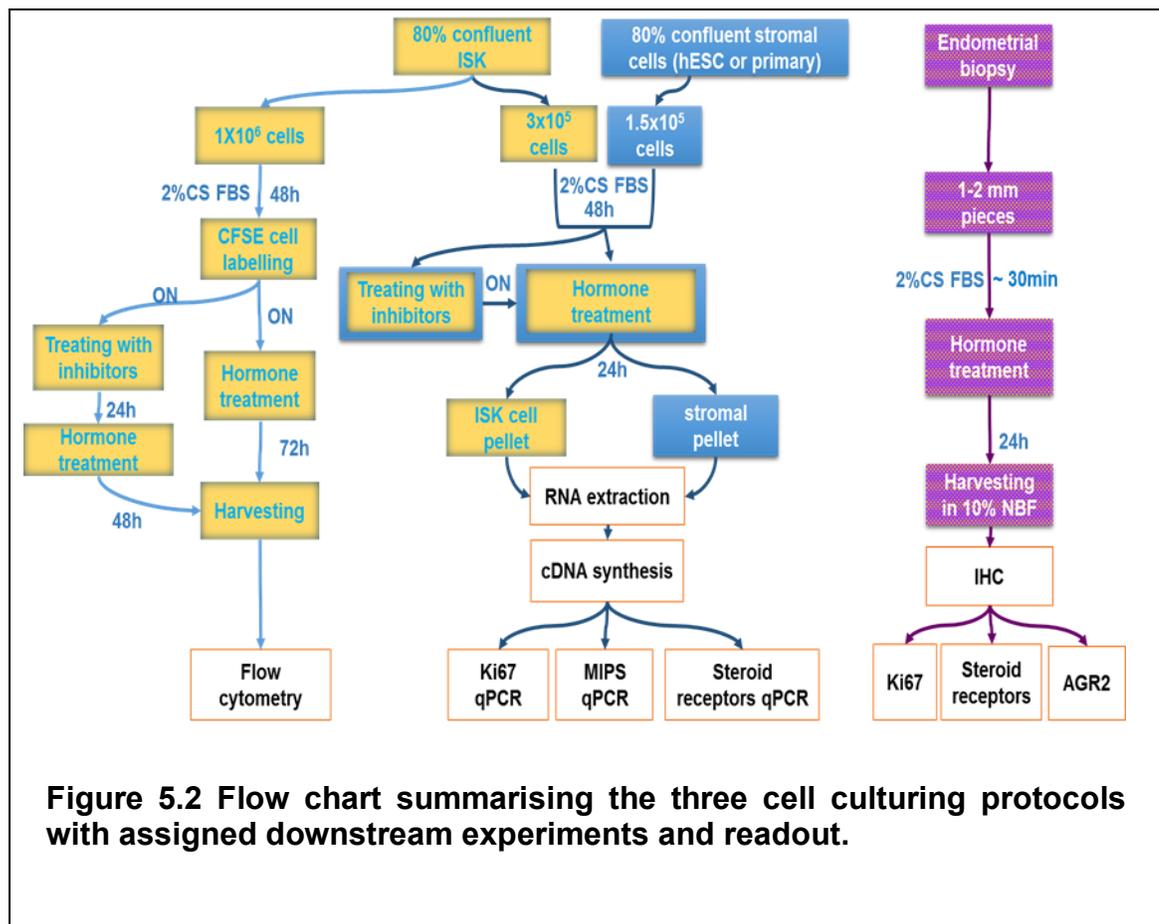


Figure 5.2 Flow chart summarising the three cell culturing protocols with assigned downstream experiments and readout.

A minimum of 30000 cells /tube were analysed by flow cytometer (Beckman Coulter Cytomics FC-500, High Wycombe, UK). The resulting data were then analysed by the aid of public domain Cyflogic software.

The cells were first gated on FSC (forward scattering) and SSC (side scattering) to identify the epithelial cell population. Percentages of proliferating cells treated with each condition were gated compared to the vehicle control.

5.3.5 cDNA preamplification

The amplification of ER β cDNA in all examined cell lines and human tissue was consistently late (after the 35th cycle). The low ER β transcript level in endometrial cells has been explained by the relatively stable expression of its protein (Hapangama et al., 2015). It is well established that at low target levels the accuracy of measurement is sensitive both to target losses during sample preparation and to high levels of sampling variability. Bio-Rad preAmp is optimised for unbiased target-specific preamplification of as little as 100 pg of nucleic acid. Importantly, it maintains patterns of gene expression changes across samples similar to standard qPCR, without the pre-amplification step (Okino et al., 2016). Following manufacturer's protocol and as shown in Figure 5.3, preAmp was performed with prime ESR2 assay (unique assay ID: qHsaCED0044944, Bio-Rad Laboratories, Hercules, CA) by diluting 5 μ l of the primer in 495 μ l NF H₂O to prepare the primer pool. Stock cDNA samples were subsequently pre-amplified for 12 cycles (Activation at 95 °C for 3 min, denaturation at 95 °C for 15 sec and annealing at 58 °C for 4min) using SsoAdvanced PreAmp Supermix (Cat# 1725160, Bio-Rad Laboratories, Hercules, CA), diluted to 5-fold and stored at -20 °C. Master mix preparation steps are summarised in Table 5.1. Target gene

was quantified by qPCR using SsoAdvanced Universal SYBR Green Supermix (Cat# 1725270, Bio-Rad Laboratories, Hercules, CA) and CFX Connect Real-Time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA) using 10 μ l reaction volumes. A four point standard curve determined the efficiency of ER β primer to be 90.3%.

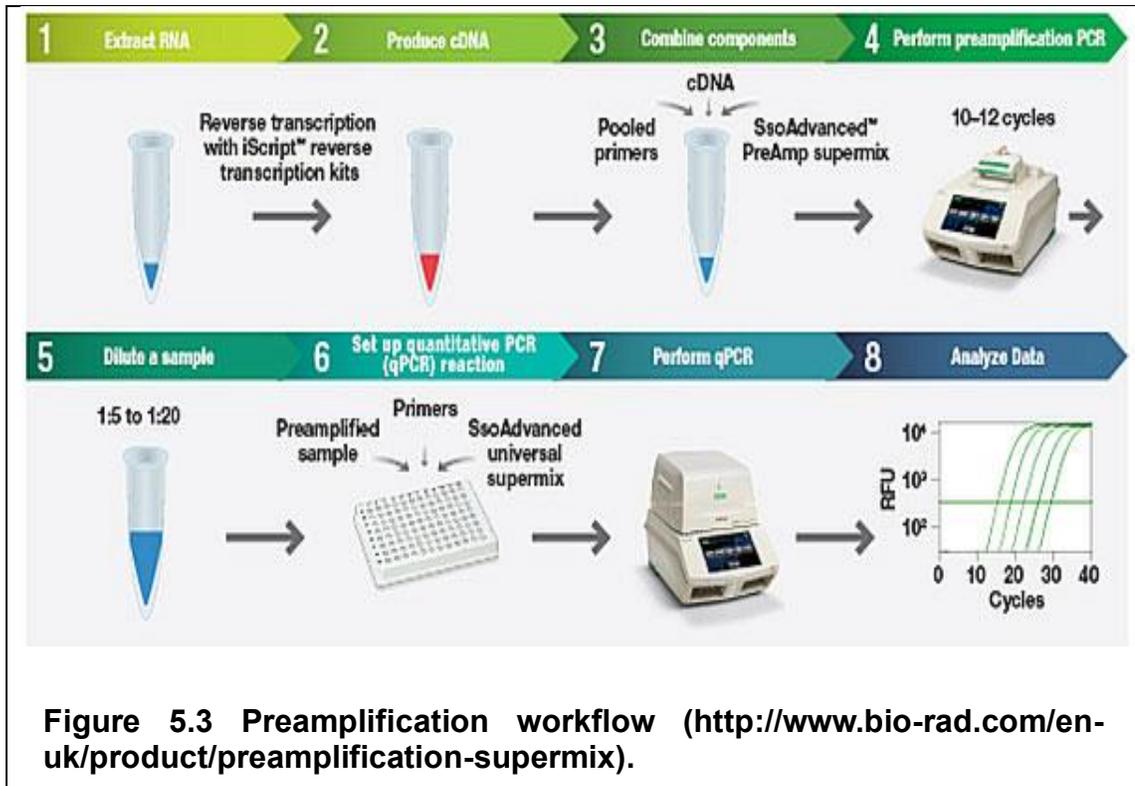


Figure 5.3 Preamplification workflow (<http://www.bio-rad.com/en-uk/product/preamplification-supermix>).

Table 5.1 PreAmp master mix

Reagents	X1
Ab pool	2 μ l
preamp mix 2X	10 μ l
NF H ₂ O	3 μ l
Template	5 μ l (~10ng RNA)
Total	20μl

5.3.6 RT-qPCR

Total RNA from tissue samples was extracted as described previously chapter 2.8 (using TRIzol® Plus RNA Purification System (Life Technologies, Paisley, UK), and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Total RNA was reverse transcribed using AMV First Strand cDNA synthesis kit (New England Bio Labs, Hertfordshire, UK) after DNase treatment (DNase I (#M0303), New England Bio Labs, Hertfordshire, UK), using the manufacturer's protocol. cDNA was amplified by RT-qPCR using JumpStart SYBR Green supermix (Sigma, Dorset, UK) and CFX Connect Real-Time System (Bio-Rad, Hertfordshire, UK). Primers are listed in Table 3.3 and Table 4.3. Relative transcript expression was calculated by the $\Delta\Delta CT$ method, normalised to the geometric mean of reference gene YWHAZ (Sadek et al., 2012) and PPIA (Romani et al., 2014) using Biogazelle qbase+ software (Biogazelle NV, Zwijnaarde, Belgium)

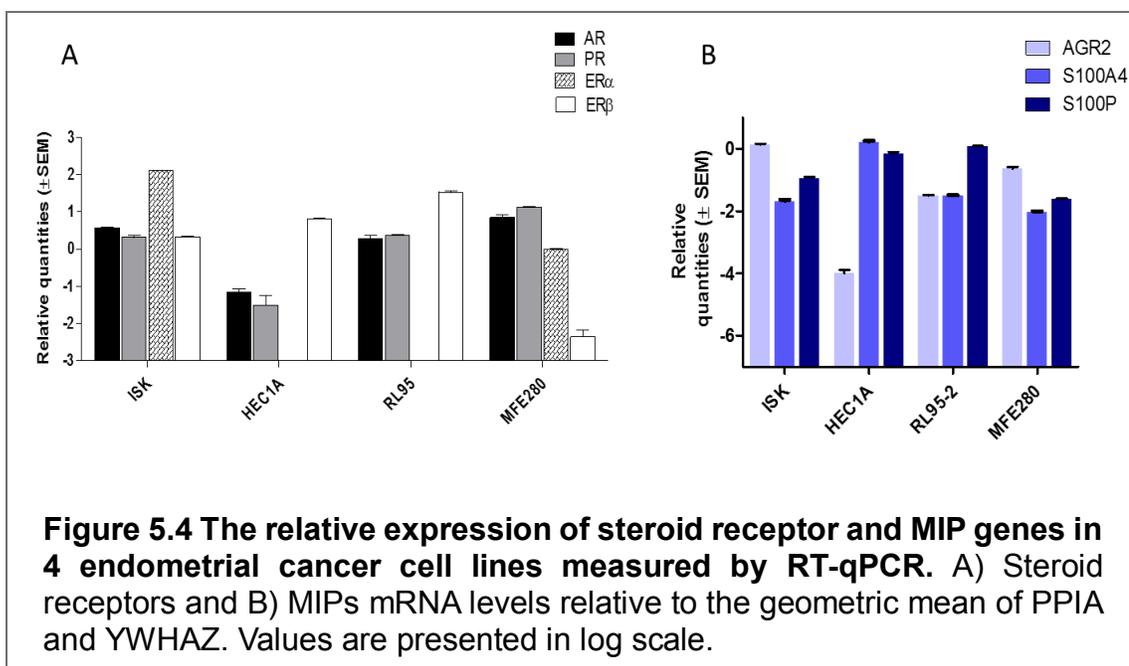
5.3.7 Immunohistochemistry

Three micron formalin fixed paraffin embedded tissue sections were immunostained with anti-human antibodies raised against steroid receptors, AGR2, and Ki67, after antigen retrieval at pH6 as previously described 2.7.1.1; antibody sources, concentrations, and incubation conditions are detailed in Table 3.2 and Table 4.2. Detection was with the ImmPRESS polymer based system and visualisation was with ImmPACT DAB (Vector Laboratories, Peterborough, UK) used in accordance with the manufacturer's instructions. Sections were lightly counterstained in Gill 2 Haematoxylin (Thermo Scientific, Runcorn, UK), dehydrated, cleared and mounted in synthetic resin. Matching isotype (0.5 $\mu\text{g}/\text{mL}$) replaced the primary antibody as a negative control, with internal positive controls performed in each staining run. Immunostains for steroid receptors, MIPS and Ki67 were analysed as previously described in 2.7.1.2 and 3.2.2.

5.4 Results

5.4.1 Endometrial cell lines differentially express steroid receptors and MIPs

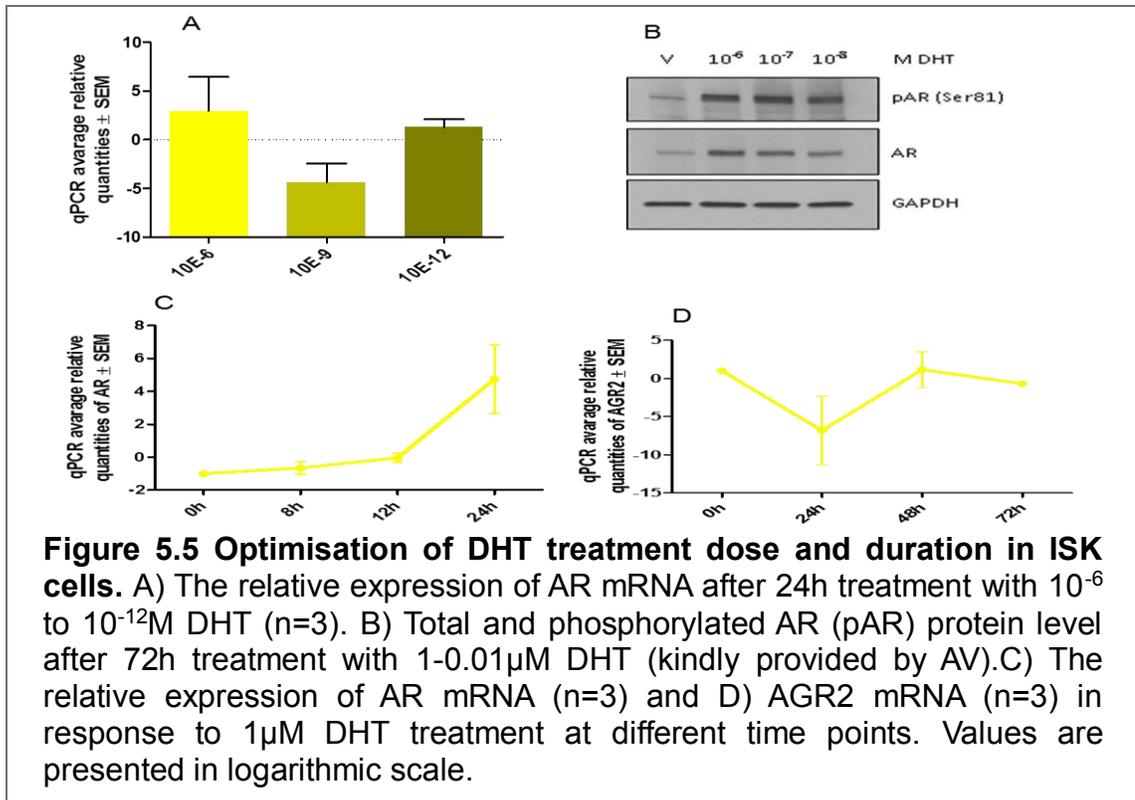
The *in vitro* assessment of the modulatory effect of steroid hormones on steroid receptors and MIPs genes expression requires the full characterisation of the endometrial cancer cell lines studied. Therefore, four different endometrial cancer cell lines that were available to us, ISK, HEC1A, RL95-2, and MFE280 were initially examined for the expression of target genes (Figure 5.4). ISK cells showed abundant ER α , as well as the other 3 steroid receptor types and a relatively high mRNA expression of the three MIPs, Figure 5.4A. In contrast, MFE280 cells which are derived from a recurrent, grade 3 endometrioid EC, showed low ER α and ER β transcripts with high AGR2 mRNA. S100A4 transcript was high in the four cells lines tested compared with S100P and AGR2 transcripts. ER α mRNA was not detected in HEC1A and RL95-2 cells however both cell lines expressed relatively high ER β transcript. This was also associated with relatively lower AGR2 mRNA in the 2 latter cell lines. Notably, the level of AR mRNA was similar to that of PR in all four cell lines, both of which were relatively high in ISK, RL95-2 and MFE280 whereas lower levels were observed in HEC1A cells. The expression of steroid receptors in ISK cell lines was similar to that of LGEC in human samples, therefore was chosen to conduct hormone modulation experiments.



5.4.2 Optimisation of DHT dose and incubation

To examine the maximum effect of DHT on gene transcription, we aimed initially to find the dose which induced the highest level of AR gene expression, in the hormone responsive, AR expressing ISK cell line. For that, ISK cells were co-cultured with hESC and treated with a wide range of DHT concentrations (10^{-6} to 10^{-12} M) for 24h and AR mRNA levels were compared with RT-qPCR. Treatment with $1\mu\text{M}$ DHT showed the highest level of AR mRNA in ISK cells (Figure 5.5A). In agreement with the above, there was a dose-dependent increase in total and phosphorylated AR protein after 72h of treatment with a small range of concentrations (0- $0.01\mu\text{M}$) (data were provided by AV, Figure 5.5B).

We next sought to determine the duration at which AR transcript levels starts to change in response to DHT treatment. ISK (co-cultured with hESC) cells were treated with $1\mu\text{M}$ DHT for 9, 12 and 24h. As shown in Figure 5.5C, the highest AR transcript level within the tested time points was achieved after 24h.



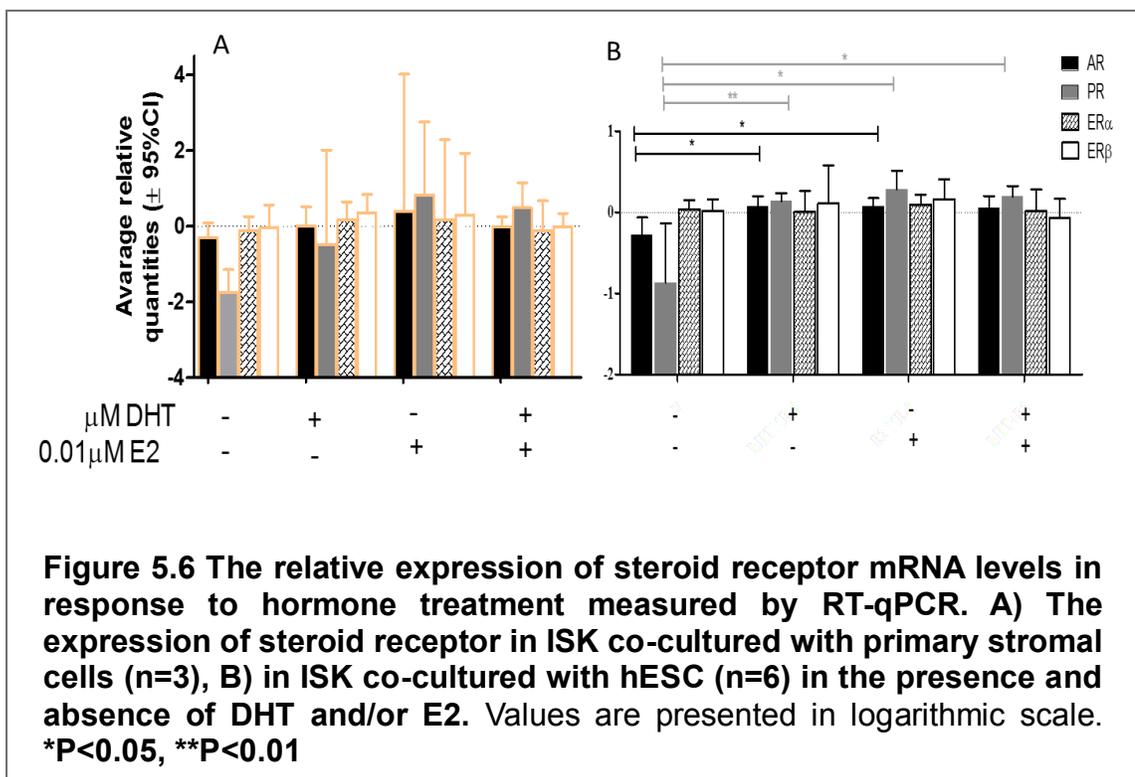
Time course experiment was conducted to determine the duration DHT may require to modulate downstream transcription of genes such as AGR2, co-cultured ISK cells were treated with 1 μ M DHT, for 24-72h. As illustrated in Figure 5.5D, AGR2 transcript levels started to change at 24h, and return to baseline at 48h.

From the experimental evidence presented above, we concluded that 1 μ M DHT and 24h incubation is the optimum conditions to induce AR gene (mRNA) and possibly similarly modulate the associated genes. Thus, these optimised conditions were used in ISK cells co-cultured with hESC for all the following qPCR experiments described in this chapter unless otherwise specified.

5.4.3 DHT modulates steroid receptor expression in ISK cells

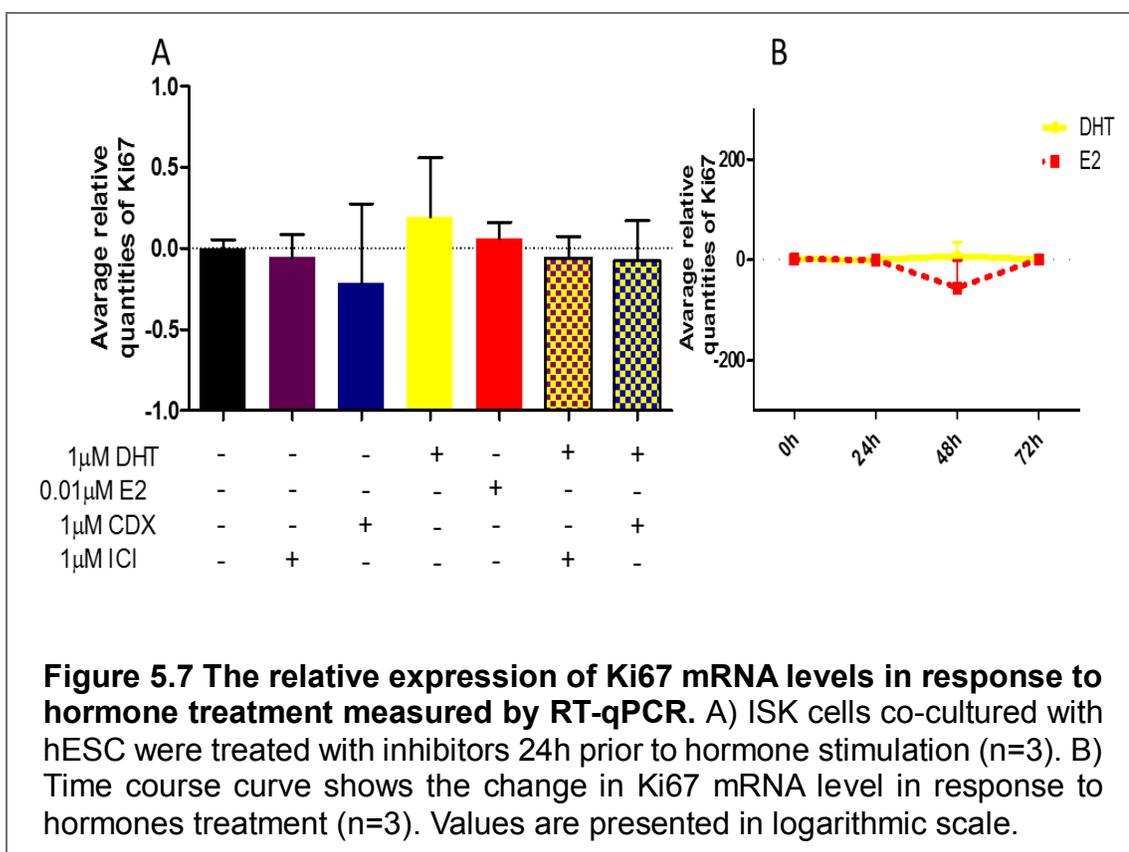
Since they are postmenopausal, androgen is the dominant circulating sex steroid hormone in most EC patients. The effect of DHT, which is not aromatised to oestrogens, on cellular steroid receptors gene expression was next investigated

in ISK cells co-cultured with hESC or primary stromal cells. The effect of DHT was considered parallel to the effects seen with the treatment of E2 (Figure 5.6). This was relevant because oestrogens are the other hormone available to endometrial cancers in the postmenopausal period, and E2 is already known to upregulate most steroid receptor expression in the endometrial cells. ER α and ER β mRNA levels were hardly affected by the treatment with the 2 hormones used. Both AR and PR mRNA however, showed significant upregulation in response to both DHT and E2 treatment alone (Figure 5.6B). Combined DHT and E2 treatment also maintained a high level of AR and PR mRNA expression levels, however, only the latter showed a statistically significantly upregulation compared with the vehicle. Similar pattern of steroid receptors expression was obtained when ISK cells were co-cultured with primary stromal cells yet variation within each target was higher compared to ISK co-cultures with hESC (Figure 5.6A).



5.4.4 The level of Ki67 mRNA in ISK cells

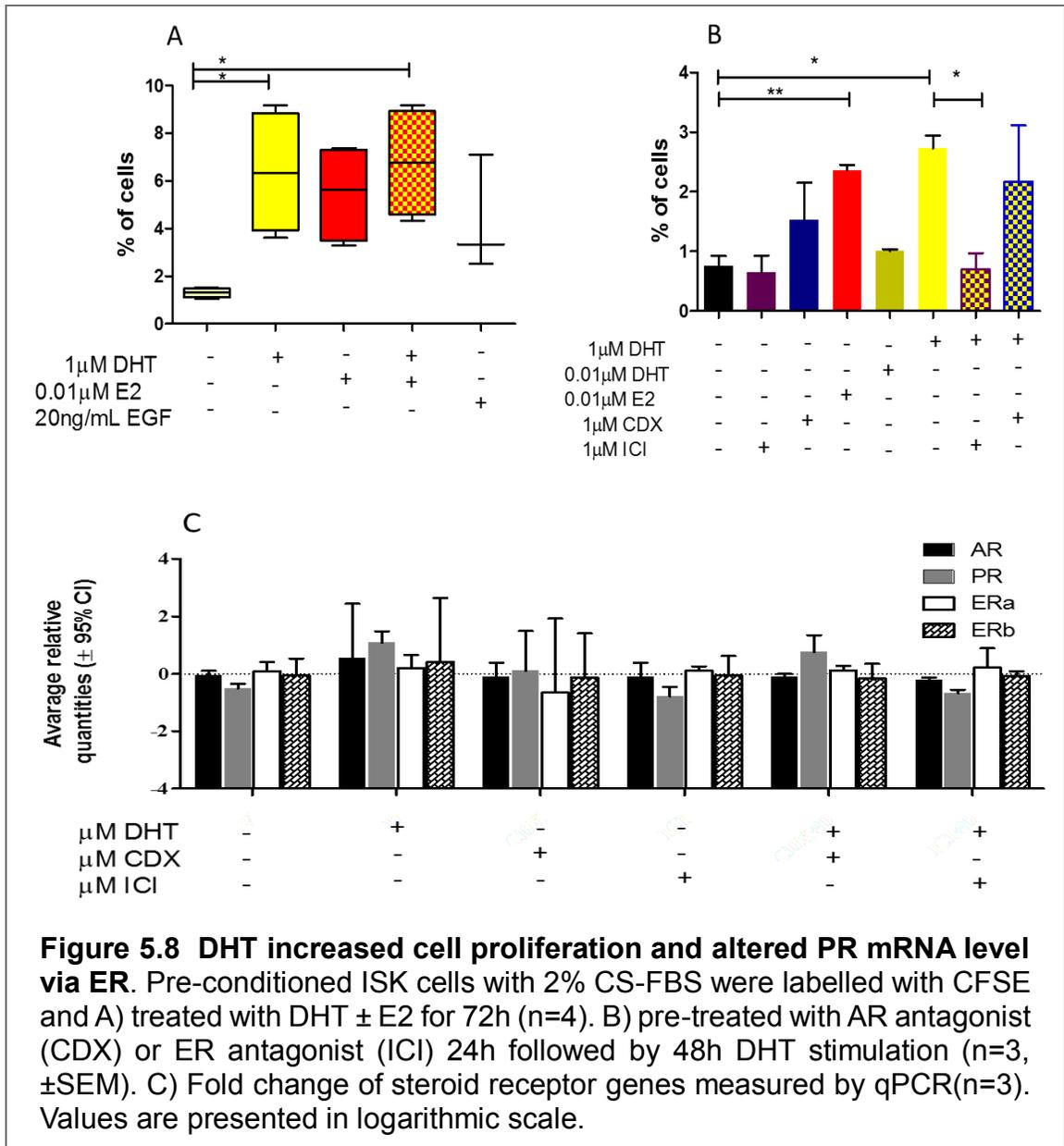
Relative Ki67 mRNA levels were investigated in hormone-treated ISK cells to see if AR and PR transcript upregulation was associated with a change in the cell proliferation levels. No significant change in Ki67 transcript level was observed in treated cells when compared with a control vehicle (Figure 5.7A) Several time-points were considered none of which showed alteration in Ki67 transcript (Figure 5.7C) which could be due to high basal Ki67 expression in the ISK cells. Therefore, an alternative proliferation assay (CFSE proliferation assay) was considered.



5.4.5 DHT induces cell proliferation via ER

Pro-proliferative effects of E2 on endometrial epithelial cells are well established and are thought to be exerted via its cognate receptor, ER α . Whether DHT affects the well differentiated ISK cell proliferation (containing functional steroid receptors akin to normal endometrial epithelial cells) was the next question. ISK monocultured cells preconditioned with 2% CS-FBS were stimulated by epidermal growth factor (EGF), E2 or DHT for 72h after labelling with CFSE. All three treatments induced cell proliferation; however, only cells treated with DHT alone or combined with E2 showed significant proliferation compared to vehicle control (Figure 5.8A). To investigate whether this stimulation was through AR, ISK monocultured cells were treated with 1 μ M AR antagonist, CDX, or ER antagonist, ICI182,780, 24h prior to DHT treatment. Intriguingly, CDX only partially reduce proliferative effect whereas ICI completely restored basal proliferation levels suggesting that excess DHT might be converted to an oestrogenic compound especially as a lower DHT concentration (0.01 μ M) did not induce cell proliferation as shown in Figure 5.8B.

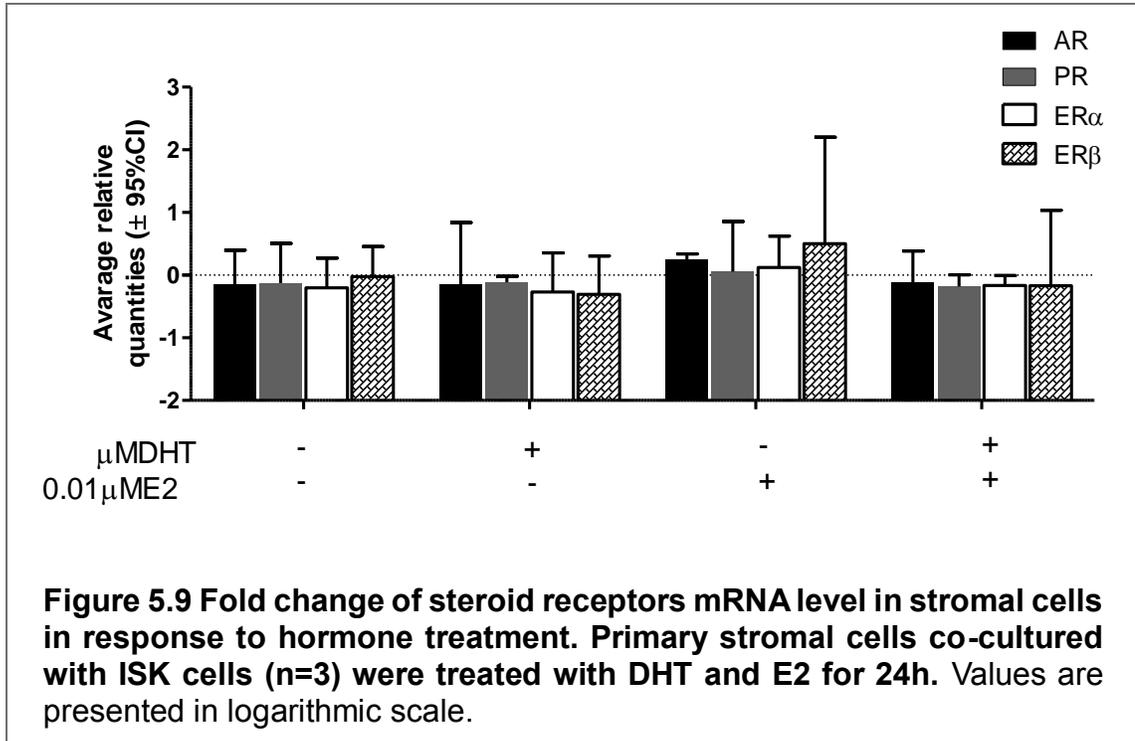
Steroid receptor mRNA was further assessed to explore whether the observed DHT-induced upregulation of AR and PR was through AR directly or an indirect effect through ER. As illustrated in Figure 5.8C, in a co-culture system, pre-treating with CDX restored AR basal level, although it did not affect PR upregulation. By contrast, pre-treating with ICI blocked the DHT induced upregulation of both AR and PR. ER α and ER β mRNA levels did not show any change in response to DHT treatment in the presence or absence of the inhibitors.



5.4.6 The expression of steroid receptors in primary endometrial stroma is regulated by oestrogen

The effect of hormone modulation on stromal steroid receptors expression was also investigated. All four steroid receptors mRNA in primary stromal cells co-cultured with ISK tend to increase in response to E2 treatment, whereas no

obvious change was observed with DHT treatment. Furthermore, DHT antagonised E2 effect on steroid receptor mRNA when combined treatment was added as shown in Figure 5.9.

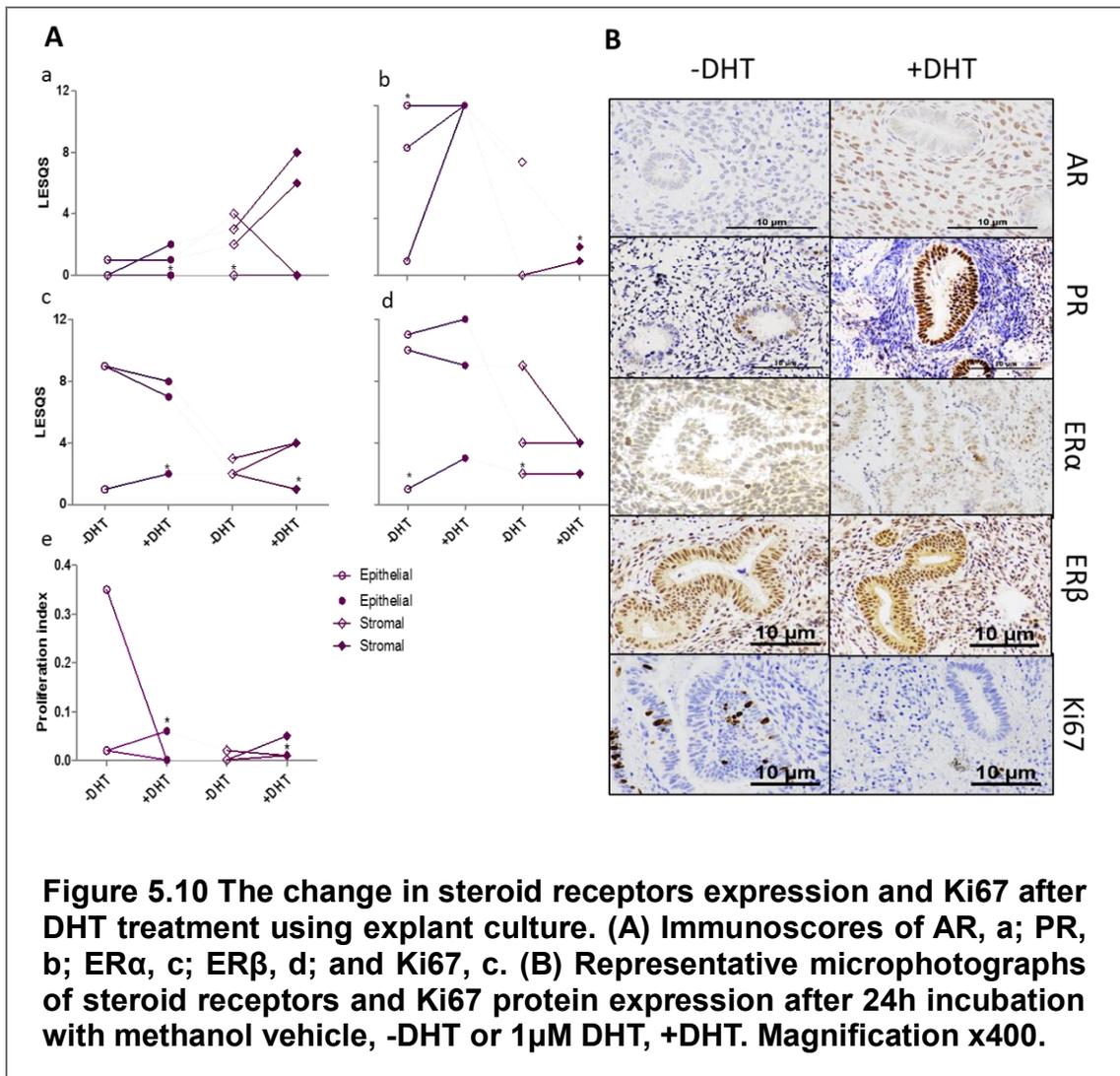


5.4.7 The effect of DHT treatment on explant culture

The effect of DHT on endometrial epithelial cell proliferation and their steroid receptor expression was further investigated in an endometrial explant culture model which preserves an intact 3D epithelial-stromal architecture thus permitting cellular crosstalk.

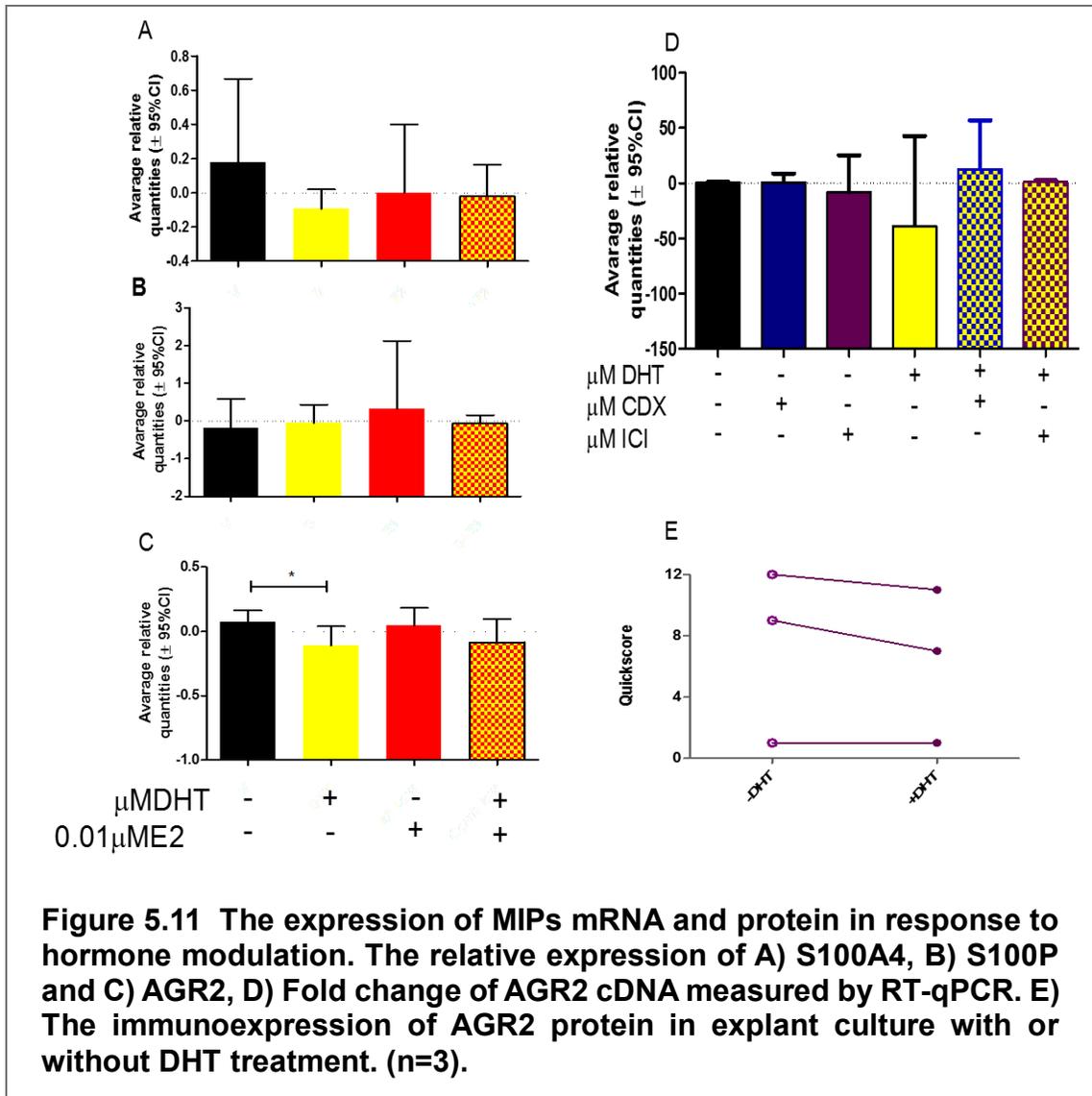
24h DHT treatment of tissue explants tend to increase PR and ERβ expression in the epithelial cells and reduce ERα with no obvious change in the AR. In the stromal compartment, DHT treatment increased AR in 2/3 PP samples whereas in SP samples there was a complete lack of immuno-reactivity to AR in DHT-/DHT+ (Figure 5.10B). In 2/3 explants, there was a slight increase in stromal ERα

whilst the remaining sample showed a reduction in ER α as shown in Figure 5.10A. The change in the proliferation index with DHT treatment was more prominent in the epithelial compartment, where a decline in proliferation was observed in 2/3 explants Figure 5.10Ac and B. This experiment provided data in favour of an anti-proliferative effect of DHT in normal endometrial tissue even when the nuclear AR was absent in the epithelial cells; however, expanding the number of samples in the future would confirm the reproducibility and draw firm conclusions.



5.4.8 Steroid hormones regulate AGR2 but not S100 proteins

MIPs mRNA levels were quantified in co-cultured ISK cells after treatment with hormones to study their effects on protein expression in human endometrial tissue. Both of the S100 proteins did not show significant changes after hormone treatments, although a slight (not statistically significant) decrease was observed in the S100A4 transcript in DHT treated cells (Figure 5.10A and B). On the other hand, AGR2 mRNA level showed a significant reduction after DHT treatment ($P=0.0313$), which was restored with the addition of the AR antagonist (Figure 5.10C and D). E2 treatment on its own preserved a basal AGR2 level, however, when E2 was combined with DHT treatment, the net result was an apparent reduction in AGR2 mRNA levels ($P=0.056$) (Figure 5.10C). Similar results were also seen in the explant culture system and there was a slight reduction in AGR2 mRNA levels observed after treating the explant with DHT, yet statistically this observation was not significant (Figure 5.10E).



5.5 Discussion

This chapter provides new insight into the complex effect of the most potent naturally occurring androgen, DHT, on human endometrium. In this context, the experimental evidence was focussed on the hormone regulation of steroid hormone receptor expression, cell proliferation and the modulation of the MIPs genes in the endometrium.

Prior evidence suggested that epithelial and stromal cell cross talk plays an essential role in neoplastic transformation of hormonally regulated tumours, including EC (Cooke et al., 1997; Arnold et al., 2001). Therefore, ISK cells which expressed relatively equivalent levels of steroid receptors (at both mRNA and protein levels) were chosen for our studies. ISK cells were then co-cultured with endometrial stromal cells allowing some inference between the two cell systems through the soluble-paracrine signals. Furthermore, the chosen co-culture model preserved cell purity as well as allowing the assessment of the transcript levels for steroid receptors in response to hormone modulation in both cell types at the same time. ISK co-culture with immortalised hESC showed a similar hormone modulation, as observed by assessing the level of the steroid receptor mRNA expression to that obtained with the primary stromal cells. A major challenge with primary stromal cells was interindividual variation due to cycle phase and age. In clinical setting, sampling of the endometrium cannot be timed to research requirements. Moreover, the results from histological menstrual cycle dating are usually not available at the time of stromal isolation. The immortalised cell line evidently displayed less variation; therefore, immortalised hESCs were preferentially used for the remaining experiments.

DHT was selected to activate AR in our studies based on two specific reasons. First reason was, unlike testosterone, DHT is not subjected to an aromatisation, and thereby its conversion to E2 and subsequent oestrogenic action could be avoided. Secondly, DHT has a superior binding efficiency to AR, and the DHT/AR complex has a longer half-life (~2.5 longer than that of Testosterone/AR) resulting in a sustained and efficient AR signalling (Sedelaar and Isaacs, 2009). Circulating DHT levels in women of reproductive age is as low as 0.06 nM, and that is about 30-50% of the level of their circulating testosterone (Arlt et al., 1998). However, importantly, DHT is also produced locally in peripheral tissues such as in the endometrium by irreversible reduction of testosterone catalysed by 5 α reductase enzyme (Ito et al., 2002; Tanaka et al., 2015) as illustrated in Figure 5.1. In EC, approximately a 10-fold increase in local DHT production has been recently reported and was significantly associated with an increased 5 α reductase enzyme

level (Tanaka et al., 2015). When *in vitro* hormone modulation is considered, the literature is inconsistent regarding the optimum dose of DHT to be used in *in vitro* studies using endometrial cell lines to simulate the *in vivo* situation. A wide range of concentrations (1 μ M-10pM) had been used reporting measurable hormonal response (Hackenberg et al., 1994; Tuckerman et al., 2000; Apparao et al., 2002). Aiming to achieve the maximum activation of AR, we examined the response to a range of DHT concentrations in our model system and found that 1 μ M dose, which is slightly higher than the physiological dose of DHT, induced the highest AR transcript activation. This treatment was also associated with an increase in total and phosphorylated AR protein levels. Interestingly, AR mRNA dose response was biphasic; an increased message was seen at 1 μ M DHT and also at a sub-physiological dose of 1pM, whereas the lowest level of AR mRNA level was induced by the supposedly physiological dose of 1nM. This data may correspond with the low/absence of AR protein immunoexpression observed in the premenopausal human samples,

Data presented here describe the regulatory role of DHT on AR, PR, ER α and ER β expression simultaneously for the first time. Furthermore, these effects are presented in comparison to the observed effect of E2, using an *in vitro* co-culture of cell lines model and an explant culture model. We have shown that DHT not only significantly increased AR mRNA expression but it also upregulated PR. ER-mediated upregulation of AR was previously confirmed by the experimental evidence showing a complete abrogation of E2 effect with the addition of ER antagonist (Apparao et al., 2002). Likewise, we have shown that direct upregulation of AR mRNA by DHT was inhibited with CDX, confirming similar finding on protein level by Apparao et al in 2002. Interestingly, this DHT effect on AR was also abrogated by fulvestrant, an ER antagonist. Furthermore, we have also shown for the first time that upregulation of PR by DHT was mediated through ER, not AR. Furthermore, it was of particular interest that ISK cell proliferation induced by DHT was, in fact, a function exerted via ER. As previously mentioned, DHT is a non-aromatized androgen, therefore the effect of DHT on ER should be due to an alternative oestrogenic metabolite of DHT. The enzyme 17 β -

hydroxysteroid dehydrogenases (HSD17B7), can reduce DHT to 3 β -androstenediol (Adiol), and this metabolite of DHT has high affinity to ER (Rižner, 2013). HSD17B7 enzyme is known to be expressed by ISK cells (Hevir-Kene and Rizner, 2015). Elevated alkaline phosphatase, a surrogate of ER receptors activation, was reported to be present in ISK cells previously after treatment with 1 μ M DHT (Holinka et al., 1986). However, in the absence of ER, the proliferation of MFE285 endometrial cancer cell line was inhibited by DHT (Hackenberg et al., 1994). Taken together, we conclude that DHT in supra-physiological doses (1 μ M) and in the presence of the specific catalysing enzyme (as in the ISK cells) can be converted to oestrogenic compounds. These compounds, in turn, activate oestrogen receptors, modulate steroid receptor expression and promote cell growth and proliferation. Fulvestrant is a selective ER α antagonist in breast tissue; however, ER β antagonism has also been demonstrated in ER α / ER β expressing cells such as U2OS, an osteosarcoma cell line (Evers et al., 2014). Although Adiol was shown to have a higher affinity to ER β in studies of prostate tissue (Weihua et al., 2000, 2002), an agonistic effect on ER α has also been demonstrated in breast tissue (Miller et al., 2013). It is widely accepted that when both ER isoforms are present in the endometrial cells, ER β counteracts the effects of ER α (Hapangama et al., 2015) which may suggest that the observed ISK proliferation in response to DHT is likely to be via ER α .

The regulation of human endometrial stroma by steroid hormones is clearly evident from the cyclic changes in stromal cell morphology, function and steroid receptor expression in premenopausal women. Androgen involvement in endometrial stromal cell decidualisation has been widely reported by many authors (Iwai et al., 1995; Apparao et al., 2002; Maliqueo et al., 2003; Cloke et al., 2008; Gibson et al., 2013). Direct androgenic effects through AR has been associated with transcriptional changes in the decidualised cells (Cloke et al., 2008), nonetheless, *in situ* conversion of testosterone to oestrogen has also been suggested (Gibson et al., 2013). In primary stromal cell monocultures, treatment with testosterone reduced the AR, PR and ER expression (Iwai et al., 1995) whereas E2 treatment was associated with a slight increase in AR protein level

(Apparao et al., 2002). We have shown that steroid receptor expression in primary stromal cells, that were co-cultured with ISK were mainly under the control of E2. Expression of the four ovarian hormone receptors studied was stimulated by E2 treatment (via ER) in the endometrial stromal cells whereas no clear change was seen with DHT. Although increased aromatase activity had been observed in decidualised stromal cells favouring an oestrogenic milieu (Tseng et al., 1986; Gibson et al., 2013), in our co-culture model, all the stromal cells used were isolated from either the late proliferative or the early secretory phase endometrium and we did not attempt *in vivo* decidualisation. ISK cells are capable of reducing DHT to oestrogenic compounds and these metabolites are likely to be also available to the stroma via diffusion, yet in contrast to the observation in ISK cells, no difference in steroid receptor expression was seen in stroma with the DHT treatment. By contrast, DHT reversed the E2 effect on stromal steroid receptors expression. This suggests that DHT has a cell-type specific action in the endometrium.

The effect of DHT on modulating steroid receptors was also examined in endometrial explants, which biologically represent a more relevant model with the maintenance of 3D tissue architecture and communication between cells. Taking into consideration the small number of samples analysed and variation in the phase of the cycle, the role of chance cannot be excluded. The description of observed trends was intended to identify the feasibility of using this model in studying changes in proteins in response to hormonal stimuli. Generally, 24h treatment with DHT was barely enough to show slight changes in the expression of steroid receptors (AR, PR ER α and ER β); however, when the receptor is not expressed, 24h treatment was not enough to induce its expression. For instant, AR is not expressed by premenopausal glandular epithelium, and after 24h of DHT treatment of the explants, the change in AR was only observed in stromal cells which already express AR, whilst no change was seen in epithelial AR expression. PR protein immunoexpression tends to increase in glandular epithelium with DHT consistent with PR mRNA response in ISK. Interestingly, while ERs did not show any change in response to DHT on mRNA level in ISK

cells, a trend to reduce expression was observed in ER α with a simultaneous increase in ER β protein immunorexpression in the explants. Consistent findings on transcript level were reported in explants treated with androstenedione (Maliqueo et al., 2003). Lower ER α /ER β ratio due to ER β dominance may explain the antiproliferative effect of androgens in normal endometrium. Antiproliferative effects of androgen, specifically androstenedione, have been previously demonstrated in normal premenopausal primary cells, however, a similar response could not be replicated with other androgens like DHT, testosterone or DHEA (Tuckerman et al., 2000). The difference we observed between normal epithelial and ISK in the proliferative response at a similar dose of DHT could be due to the absence of reducing enzymes in normal tissue which in ISK cells convert DHT to oestrogenic compounds.

To confirm IHC results discussed in chapter 4, we explored hormonal modulation effects on MIPs. The literature is inconsistent regarding oestrogen regulation of S100A4 (Xie et al., 2007; Chong et al., 2014). We did not observe any correlation between S100A4 expression and the expression of the steroid receptors in human endometrial samples. Therefore, we sought to further confirm that in ISK cells. Although S100A4 mRNA level was slightly lower after E2 and DHT treatment, it was not significant. Likewise, S100P mRNA levels did not show a significant change in response to hormone modulation. AGR2 message, on the other hand, was downregulated by DHT while E2 treatment failed to change the basal level of AGR2. We have shown in chapter 4 that the prognostic effect of AGR2 immunorexpression was dependant on the steroid receptor status of ECs suggesting a different function of AGR2 in the presence of different combinations of steroid receptors. In our *in vitro* ISK cell line model which expressed all the steroid receptors, DHT treatment downregulated AGR2 transcripts. In the same model, DHT induced proliferation of ISK cells via ER and upregulated AR. ER and AR antagonists restored the basal level of both AGR2 and AR. Oestrogen and androgen receptor binding sites have been localised to the promoter of AGR2 (Hrstka et al., 2010; Bu et al., 2013). Furthermore, when the AGR2 promoter was cloned upstream of a luciferase reporter and transfected with ER α in ER α /AGR2

negative cells, activation of the luciferase reporter by ER α was minimally induced by E2 or tamoxifen (Hrstka et al., 2010), which may suggest the involvement of other steroid receptors in AGR2 regulation such as AR or ER β . The level of AGR2 mRNA in endometrial cell lines appeared to be independent of nuclear ER α expression. HEC1A and RL95-2 cells which completely lack ER α , but express ER β , still express AGR2 suggesting that, unlike breast, oestrogen might regulate AGR2 via ER isoforms other than ER α . Induction of AGR2 by tamoxifen has been suggested as a plausible mechanism in tamoxifen resistant breast cancer (Hengel et al., 2011). The carcinogenic effects of tamoxifen in the endometrium appear to be mediated by ER isoforms (Hu et al., 2015). We have shown that in patients with low ER α /ER β ratio, AGR2 was associated with worse patient outcomes. Taking all these observations together, we can conclude that the dysregulation of ER isoforms homeostasis is likely to contribute to the ultimate function of AGR2 in EC.

Chapter six

General Discussion

6.1 Overview of research findings

The alarming increase in endometrial cancer (EC) incidence associates with a parallel increase in cancer-related deaths (Duncan et al., 2012). High grade endometrial cancer (HGEC) carries the worst patient outcomes and also associates with a high risk of therapy failure (Gadducci et al., 2011), however, the available treatment options for these patients remain limited. Although hormonal therapy has been approved for the advanced and recurrent EC, it was mainly indicated for the cancer subtype with an endometrioid histology (Colombo et al., 2015). In this respect, prediction of the efficacy of hormonal therapy with the evaluation of the tumour for steroid receptor expression and understanding the mechanisms of chemo-resistance continue to be important challenges. There is an urgent need for reliable biomarkers that can predict tumour behaviour, not only to better stratify risk groups, but also to provide new therapeutic targets. This thesis provided a comprehensive description of the expression of ovarian steroid receptors and metastasis inducing proteins (MIPs) in primary and metastatic human EC samples and evaluates their utility as diagnostic and prognostic biomarkers for EC. It further examined the role of the ovarian steroid hormone, androgen in modulating EC cells phenotype and activity *in vitro*.

6.1.1 Androgens and steroid hormone receptors in primary endometrial cancer

Ovarian steroid hormones play a prominent role in regulating normal endometrial function. The contribution of these hormones to the endometrial proliferative conditions, including cancer, has been researched for many years. Although estrogen and progesterone dominate the field of EC research, yet increasingly, this interest is directed towards androgens. Since the nonendometrioid EC were being widely accepted as hormone independent cancers, the reports describing their steroid receptor expression are limited (Alkushi et al., 2010; Mhawech-Fauceglia et al., 2013) with no previously published data on AR and ER β expression in serous, clear cell carcinomas and carcinosarcomas (Hapangama et

al., 2015); however, a recent pooled epidemiological study including an adequate number of nonendometrioid EC has shown that the latter shares similar risk factors with the endometrioid EC (Setiawan et al., 2013). To allow a panoramic vision of the functional interplay between the steroid receptors, it was compelling to characterise all subtypes of human EC for the simultaneous presence of all these receptors using the same, optimised and validated scoring method. Using immunohistochemistry (chapter 3), I have shown for the first time that the glandular epithelium of PM endometrium acquired AR whilst preserving the expression of the other steroid receptors. This finding highlights the importance of selecting a suitable control group in similar studies and further illustrates the important phenotypic similarities and differences between the postmenopausal (PM) atrophic endometrium and the basalis and functionalis layers of the premenopausal proliferative phase endometrium. Results presented in this thesis also showed that AR and PR protein immunoexpression were significantly lost in HGEC, whilst ER isoforms expression (at least low ER β) were sustained, which may suggest a potential for application of endocrine therapy in that particular group. I also confirmed that AR expression associated positively with favourable prognostic parameters and correlated with longer disease free (DFS), cancer specific (CSS) and overall (OS) survivals. The absence of both AR and PR expression in the tumours was superior to PR loss alone in predicting the patient survival and AR was an independent prognostic indicator of EC relapse. This data merits further investigation in to the potential role of AR as a marker predicting the response to endocrine therapy.

With the observed inverse correlation of AR with Ki67 in human EC samples we hypothesised that activated AR may have a suppressor effect on tumour growth and progression. I therefore examined the effect of direct activation of AR in the human endometrium by dihydrotestosterone (DHT) *in vitro*, employing the human endometrioid adenocarcinoma cell line ISK and an explant cultures of benign human endometrial tissue (chapter 5). My results indicate that a supra-physiological dose of DHT was required to induce AR expression in ISK cells. At this dose, DHT had a stimulatory effect on ISK cell proliferation, and this action

was exerted via ER. DHT is not a substrate for the aromatase enzyme and cannot be converted back to testosterone, yet, it can be metabolised to 3β -androstenediol (Adiol) compounds which have a variable affinity to ERs. This finding may also explain the observed upregulation of PR gene transcription with DHT treatment which is only reversed by the ER antagonist (Figure 6.1). Results from this piece of work may suggest that high concentrations of DHT in EC may evoke mainly oestrogenic, therefore, pro-oncogenic pathways which override the expected antiproliferative androgenic response to DHT. Involvement of DHT metabolising enzymes which can be manipulated with various pharmaceutical agents can therefore be further investigated in the future as potential therapeutic targets.

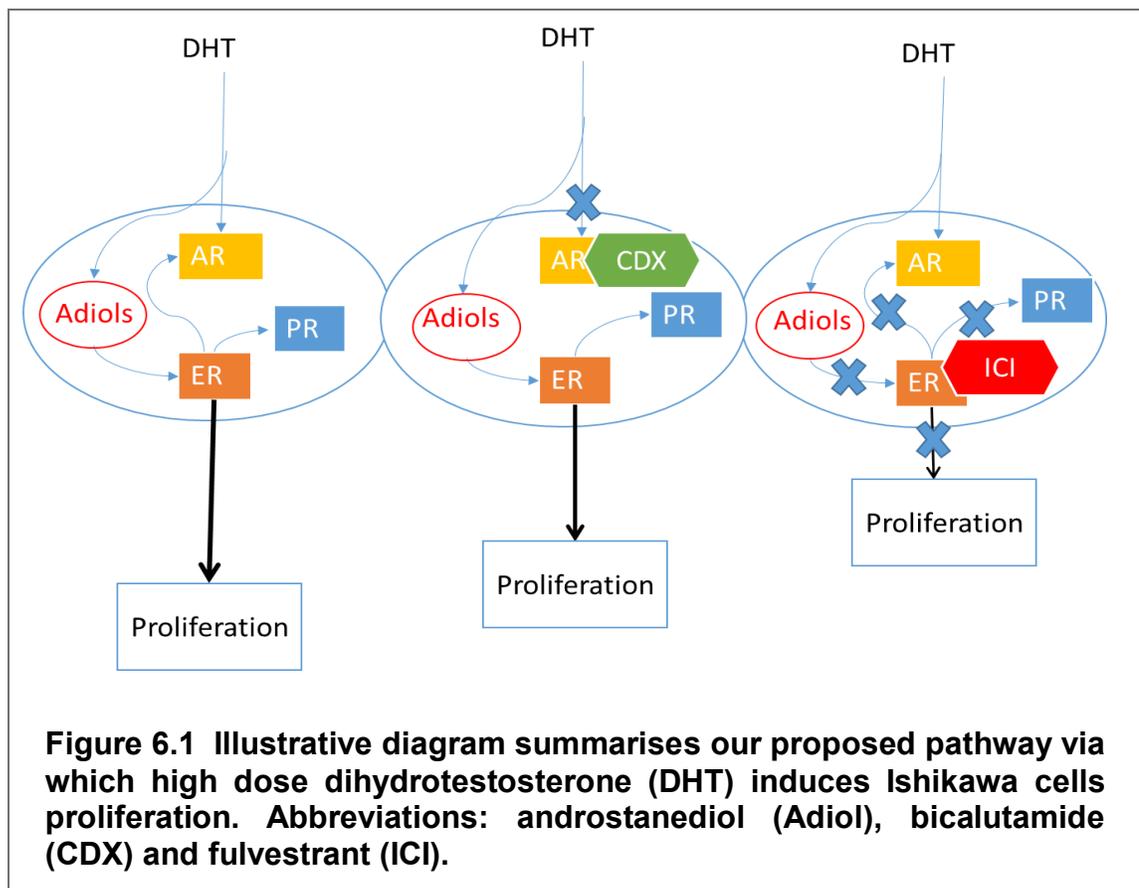


Figure 6.1 Illustrative diagram summarises our proposed pathway via which high dose dihydrotestosterone (DHT) induces Ishikawa cells proliferation. Abbreviations: androstenediol (Adiol), bicalutamide (CDX) and fulvestrant (ICI).

6.1.2 The expression of MIPs in EC and hormonal regulation

EC generally is associated with a good patient outcome compared to most other gynaecological cancers; however, recurrence is recorded in 11- 23% of cases (Fung-Kee-Fung et al., 2006). The mechanisms involved in EC spread and relapse are not well understood. The histopathological predictors of recurrence are limited to the cervical stromal invasion, lymphovascular space invasion and myometrial invasion with no reliable biomarker that can predict prognosis. The involvement of the MIPs, S100A4, S100P and AGR2 in the metastasis and therapeutic failure of the breast, prostate, and other tumours propose them as candidates to investigate for a similar role in EC. I have shown in this thesis that the three MIP proteins were upregulated in EC (chapter 4). A substantial change in the expression profile of S100A4 was observed in EC compared with the normal PM endometrium, agreeing with the previous reports. The increase of S100A4 was represented by a significant expression of the protein in the nuclei and in the cytoplasm of malignant epithelial cells with a concomitant increase in the stromal expression. This finding could be a consequence of enhanced epithelial mesenchymal transition (EMT) process in cancer cells and may be contributing to the modulation of the infiltrating lymphocytes, hence promoting EC invasion. High immunoexpression of S100A4 was positively associated with deep myometrial invasion and short CSS and OS but was not identified as an independent prognostic indicator. The expression of S100P is generally low and limited to the cytoplasm of normal PM glandular epithelium. Significant nuclear translocation was observed in EC cells simultaneously with a reduction in cytoplasmic expression. The loss of cytoplasmic S100P (not nuclear) was associated with unfavourable prognostic indicators such as lymphovascular space invasion and cervical stromal invasion suggesting different molecular functions of S100P at different cellular localisation. Hyperplastic endometrial epithelial cells characterised by the complete lack of S100A4 and S100P may suggest the involvement of these proteins in EC progression rather than initiation.

The third MIPs studied, AGR2, showed a completely different expression profile to the two S100 proteins in EC (chapter 4). Elevated AGR2 expression was observed in premalignant atypical hyperplasia (EHA) and peaked in low grade cancers (LGEC). There was a significant association between *highAGR2* and positive ER α , PR, and AR in EC patients. Secreted AGR2 was detected in the serum and uterine washes from EC patients. AGR2 protein was immunoexpressed in 93/100 (93%) of the EC samples and was associated with longer OS of EC patients. In a subset of patients with low ER α /ER β ratio, *highAGR2* was associated with a significant increase in OS which indicates the involvement of the ER isoform homeostasis in regulating AGR2 function. Furthermore, 80% of LGEC patients who had *highAGR2* immunoexpression developed a progressive disease. Although analysis of survival data was not possible for these patients because of the small number of cases in our cohort, this warrants further investigation in an appropriately powered sample to assess the prognostic value of AGR2 in this group.

Hormonal regulation of MIPs was further investigated *in vitro* (chapter 5). Whilst S100A4 and S100P mRNA level in ISK cells did not show a significant change in response to E2 or DHT treatments, AGR2 mRNA level was downregulated after 24h treatment with DHT (not E2) which was, at least partially, via AR; however, ISK cells showed a proliferative response to DHT via ER. While these results were in agreement with AGR2 expression in human endometrium in terms of association of AGR2 with better outcomes in the presence of steroid receptors, it suggests an indirect regulation of AGR2 (transcript at least) by ER isoforms.

6.1.3 Phenotypic changes in metastatic endometrial cancer

EC patients with metastatic lesions at diagnosis constitute approximately 20%, and the survival rate of these patients remains particularly low. The majority of these cases presented with regional spread with distant metastasis only reported in 7%. If surgical resection was successfully attempted, at least half of these patients are expected to develop recurrence within 3 years (Mundt et al., 2001).

Characterization of the regional metastatic lesions could provide clues for subsequent tumour recurrence, however, little is known about the phenotype of these metastatic lesions and the mechanisms behind their spread and settlement. I have shown that the protein expression differs in metastatic lesions from that of the matched primary tumour. Steroid receptor profiles of metastatic lesions showed low PR protein expression compared to matched primaries, (chapter 3) which may explain the resistance observed in more than 65% of the patients to the progesterone therapy. Upregulation of AR was observed in metastatic lesions with no significant change in the metastatic cell proliferation index, which could be due to a local change in the hormonal milieu compared with that of the matched primary. MIPs expression was generally maintained in metastatic lesions (chapter 4) which may be necessary for metastatic cells survival, attachment and settlement in the new microenvironment.

6.1.4 Strength and limitations of the research

Research questions, derived from proposed hypotheses and tackling protein expression were addressed by semi-quantitative analysis. Although immunoscore was limited by observer subjectivity, I have controlled for this potential bias. Firstly, by scoring independently a set of randomly selected samples by at least two observers (interobserver reproducibility) and secondly by revisiting the samples and scoring them again by myself (intraobserver reproducibility). To compare the dynamic changes in steroid receptor expression during endometrial carcinogenesis, I used the same scoring system that was optimized according to the expression pattern of these receptors in endometrial tissue and validated this against well-established scoring systems for breast cancer.

To study the expression pattern of steroid receptors and MIPs in all EC subtypes and their prognostic utility, I have included a similar number of LGEC and HGEC. This caused our cohort to deviate slightly from the actual incidence of nonendometrioid EC, nonetheless provides a better evaluation of HGECs phenotype which is usually associated with unfavourable outcomes.

I elucidated the roles of the androgen on steroid receptors and MIPs expression and cellular proliferation of Ishikawa human endometrial epithelial cell line, using the commercially available, naturally occurring potent androgen, DHT. The direct and indirect DHT effect via AR and ER respectively was assessed by selective receptor antagonists. The authenticity of the cell line was confirmed by STR profiling. Each experiment included within this thesis was repeated at least three times and the results herein are representative of each experimental data set. Additionally, studies contained in this thesis utilise co-culture systems, which approximate better the normal biology of the uterus. Although the use of cancer associated stroma could have a better simulation to EC biology, the goal of this thesis was to study the effects of DHT on epithelial cells, unconfounded by paracrine mediated factors emanating from cancer associated stroma. Furthermore, full characterisation of endometrial cancer associated stroma and separation from adjacent normal stroma continue to be challenging.

6.2 Conclusions, impact and future directions

Throughout chapter 3 and 5 of this thesis, I have presented a case that the activation of steroid receptors, AR in particular, is an extremely dynamic and complex process. This work yielded new observations which will extend further our understanding of AR biology within human EC.

Steroid receptors status can have several implications in EC. The clinical potential of AR as a prognostic indicator of EC progression and recurrence can be further characterised in a powered prospective study targeting patients with low/moderate risk which may improve risk stratification of patients and promote individualisation of the available treatments. The prognostic value of the steroid receptor status in high risk group can be further expanded in a larger cohort including those with nonendometrioid subtypes. The presence of steroid receptors, at least ER β in this group can be a potential target for steroid hormone modulators particularly for patients who are less suitable for radio/chemotherapy. Furthermore, steroid receptor profiling of the metastatic lesions can a better predictor of

potential response to endocrine therapy compared with the receptor status of the primary tumour. This work can be further expanded and validated retrospectively in a cohort of patients who have received hormone therapy by comparing steroid receptor status of the primary and metastatic sites in relation to patient response.

Hormone modulation experiments revealed the dominance of estrogenic, pro-proliferative pathway after DHT treatment in EC. Additional studies can investigate the bioavailability and activity of the enzymes involved in DHT metabolism namely HSD17B5, 6 and 7 in EC subtypes and metastatic lesions and compare the change of their expression with normal PM and EH. Information from a study like that may further support our observations, and will be useful in developing strategies to prevent malignant transformation of the endometrium. Furthermore, specific AR modulators (SARMs) which have the advantage of specific activation of AR gene transcription without further metabolism to estrogenic compounds (Narayanan et al., 2014) can be investigated and such experiments may show whether activation of AR by SARMs continues to adhere to the overall hypothesis regarding their antiproliferative function. Further studies could also examine the role of AR on regulating genes known to contain classic AREs within their promoter to understand the functional regulation of EC cells by AR. There are several genes in which androgen response elements are characterized within their promoters. Cyclin D1 and Rb for instance are of great importance in carcinogenesis as they may play a critical role in the cell cycle progression.

Metastasis inducing proteins expression profile in human EC was detailed in chapters 4 and 5 describing the spatial and temporal expression of MIPs in endometrial tissue from normal through to the malignant transformation and progression process. The differential expression of these proteins in EC subtypes and metastatic lesions identify S100A4 and AGR2 specifically as potential prognostic indicators.

The expression of S100A4 in endometrial neoplastic cells was a late event was associated with shorter overall survival. The reciprocal expression of S100A4 in

EC cells and cancer associated stroma cells, and the association of this protein with deep myometrium invasion warrant the future investigation of the ability of a newly developed S100A4 targeted peptide to control EC invasion.

The increased expression of S100P protein in EC was significantly associated with nuclear localisation and the loss of cytoplasmic expression was associated with unfavourable prognostic parameters. The lack of nuclear S100A4 and S100P expression in EHA could be a potential diagnostic marker to differentiate them from LGEC. This could help to guide the clinical decisions to determine optimal surgical management. An adequately powered study looking into the sensitivity and specificity of these proteins individually or in combination with other known markers such as PTEN, bcl-2 or MMP-9 in differentiating EHA from LGEC could test this potential.

The expression of AGR2 protein was upregulated in LGEC. *High*AGR2 may be an indicator of overall survival of EC patients when the ratio of ER isoforms is low. The prognostic effect of AGR2 in LGEC and tamoxifen associated EC could be assessed in a suitably powered survival study. Once confirmed, the impact of targeting AGR2 (knockdown or overexpression) in cancer can be further evaluated on EC cell phenotype, proliferation and cancer signalling pathways. Downregulation of AGR2 gene expression was observed in ISK cells via both AR and ER. The importance of these results can be further dissected out by using synthetic androgens that are not metabolised in the endometrium to confirm the direct role of AR in regulating AGR2 and associated pathways.

Finally, the phenotypic profile of metastatic lesions isolated during primary surgical excision surgery could be further expanded to identify genetic signatures of these lesions compared with the matched primary tumours and the recurrent tumours. Such information will enable us to highlight how ECs evolve into a more distinct, aggressive metastatic state that should be considered for therapy development.

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Appendices

8.1 Appendix I: Ethical approvals



National Research Ethics Service
North West 2 Research Ethics Committee - Liverpool Central

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05 October 2009

Dr Dharani K Hapangama
Clinical Senior Lecturer / Honorary Consultant in Obstetrics & Gynaecology
University of Liverpool
University Department, First Floor
Liverpool Women's Hospital, Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study Title: The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis
REC reference number: 09/H1005/55
Protocol number: 1.0

Thank you for your letter of 14 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

This Research Ethics Committee is an advisory committee to North West Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Covering Letter		
REC application	2.2	
Protocol	1.0	02 July 2009
Investigator CV		
GP/Consultant Information Sheets		
Letter from Sponsor		02 July 2009
Referees or other scientific critique report		
Participant Information Sheet	2	09 September 2009
Participant Consent Form	2	09 September 2009
poster healthy + baby		
poster endometriosis		
Response to Request for Further Information		14 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

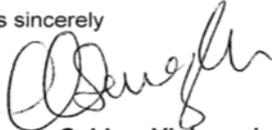
changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/55

Please quote this number on all correspondence

Yours sincerely



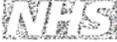
 **Professor Sobhan Vinjamuri**
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

"After ethical review – guidance for researchers"

Copy to: Mrs Gillian Vernon

Liverpool Women's 
NHS Foundation Trust

Crown Street
Liverpool
L8 7SS

Tel: 0151 708 9988
www.lwh.nhs.uk



14th April 2011

Dr Dharani Hapangama
Clinical Senior Lecturer / Honorary Consultant in O&G
Liverpool Women's Hospital
University Department, First Floor
Crown Street
Liverpool L8 7SS

Direct dial: 0151 702 4346
Email: Gillian.vernon@lwh.nhs.uk

Dear Dharani

ID: LWH0877 – Study of the Role of Metastasis Inducing Proteins and cell fate regulators in the pathogenesis of Endometrial Cancer

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by the R&D Director. This approval relates to the documentation listed below:

- Ethics approval letter [11/H1005/4] dated 11th April 2011
- Protocol [version 1.0] 9th December 2010

The research is registered on the Trust's R&D database under the reference LWH0877, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) are the Trust and the University of Liverpool.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles and guidelines set out in ICH Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf.

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely



Gillian Vernon
Research & Development Manager

Liverpool Women's 



Ethics Submission No: 09/H1005/55
 PATIENT INFORMATION SHEET
“Endometrial stem cell Study”

The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Version 1: Endometrial biopsy only

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of special cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells), and their job in the body is not yet determined. The inner-lining of the womb (endometrium) has these stem cells that can become many different types of cells, and they are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 160 women (you must have been off all hormonal medicines for at least 3 months), who have regular periods. We are specifically looking for 80 women who have endometriosis and another 80 completely healthy women who have had at least one baby. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

Hapangama / Stem cells Version 1(revision 1)

Date 09/09/09

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*

Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.

2. *If you are not having a hysterectomy:*

Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.

The sample of endometrium will be processed in the lab to isolate the stem cells from it. Therefore, **NO** extra surgery will be performed for the study. A **blood sample** (5mls = teaspoonful of) will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are in the clinic (without anaesthetic) or whilst you are under anaesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anaesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept



National Research Ethics Service

Cambridgeshire 2 Research Ethics Committee

Victoria House
Capital Park
Fulbourn
Cambridge
CB21 5XB

Telephone: 01223 597685
Facsimile: 01223 597645

18 October 2010

Dr Helen Stringfellow
Lancashire Teaching Hospitals NHS Trust
Pathology, Royal Preston Hospital
Sharoe Green Lane
PR2 9HT

Dear Dr Stringfellow

Title of the Research Tissue Bank: Archival genito-urinary tissue, blood, urine and saliva collection
REC reference: 10/H0308/75
Designated Individual: Dr Timothy P Dawson

Thank you for your letter of 30 September 2010, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 15 October 2010. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
REC application	58126/139495/3/344	30 July 2010
Covering Letter	Dr Helen Stringfellow	30 July 2010

This Research Ethics Committee is an advisory committee to East of England Strategic Health Authority.

Participant Information Sheet	2	30 September 2010
Response to Request for Further Information	Dr Helen Stringfellow	30 September 2010
Participant Consent Form	2	30 September 2010
Human Tissue Authority Licence		14 February 2008
Protocol for Management of the Tissue Bank	1	30 July 2010

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Research governance

A copy of this letter is being sent to the R&D office responsible for Royal Preston Hospital. You are advised to check their requirements for approval of the research tissue bank.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by a research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

- a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
- b) Annual Reports. Please refer to the attached conditions of approval.
- c) Amendments. Please refer to the attached conditions of approval.

This Research Ethics Committee is an advisory committee to East of England Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk

10/H0308/75

Please quote this number on all correspondence

Yours sincerely

pp Nicky Storey

**Dr Rowan Burnstein
Chair**

E-mail: Nicky.Storey@ecce.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

Standard approval conditions

Copy to: Dr Timothy Dawson
Lancashire Teaching Hospitals NHS Trust
Pathology, Royal Preston Hospital
Sharoe Green Lane
Preston
Lancashire
PR2 9HT

8.2 Appendix II: Standard operation procedures



Standard Operating Procedure 49



Liverpool Women's **NHS**
NHS Foundation Trust

STANDARD OPERATING PROCEDURE

Number	49	Version & Issue	1.0
Title	Culture of human endometrial cells		
Author	Anthony Valentijn	Date	September, 2011
Approved	D. Hapangama	Date	06.10.11
Review Date	October 2012	Last Reviewed	October 2011

Background



The endometrium is the mucous membrane that lines the uterus. It is a dynamic tissue that undergoes cycles of growth and regression with each menstrual cycle. Adult progenitor stem cells are most likely responsible for this regenerative capacity; these same cells may also be responsible for endometriosis and endometrial cancer. The endometrium becomes progressively thicker and more glandular in the later stages of the oestrous (menstrual) cycle, which prepares it for embryo implantation. If pregnancy occurs the endometrium becomes the decidua, which is shed after birth. If there is no pregnancy the endometrium returns to its original state; in primates, including humans, much of the endometrium breaks down and is lost in menstruation.

Separation of the endometrium into the epithelial and stromal components through mechanical and enzymatic (e.g. collagenase) means has permitted the study of these two cell populations. Epithelia include glandular and luminal types whilst stroma is a connective tissue comprised of predominantly fibroblasts, but also white blood cells such as macrophages and lymphocytes, and endothelial cells.

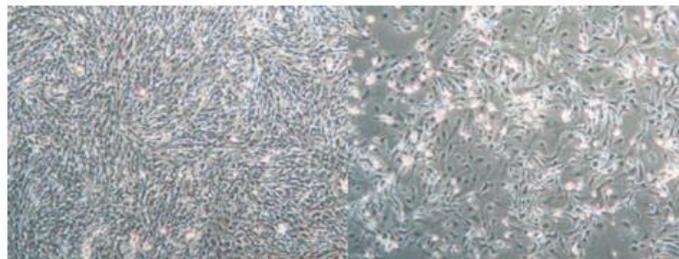
Use of human tissue is subject to the Human Tissue Act 2004 (<http://www.hta.gov.uk/>). Cells derived from human tissue that have divided in culture and therefore outside the human body are exempt from HTA. Any unused tissue must be stored according to the HTA guidelines, otherwise destroyed.

Page 1 of 7

THIS IS A CONTROLLED DOCUMENT. DO NOT COPY

Definition

This SOP will explain how to culture epithelial and stromal cells from human endometrium. Briefly, tissue is minced with sterile scalpels and then digested with collagenase and dispase. The digest is then sieved; epithelial glands are retained by the filter and stromal cells pass through. Epithelial glands are further digested with trypsin to single cells and small cell clusters. Enrichment for both cell types is achieved by selective adherence to culture dishes, with the stromal cells adhering quicker than the epithelial cells.



STROMA

EPITHELIA

Health and Safety Precautions

Please refer to COSHH and Risk Assessments pertinent to this procedure before proceeding.

All un-fixed human tissue and bloods and their primary containment should be treated as potentially hazardous material and appropriate protection and decontamination procedures must be employed. The wearing of a lab coat and gloves when handling human material is mandatory. Human material is processed in a designated Class II hood/ Biosafety cabinet (see below). Scalpel blades are used to chop. Care should be

taken to avoid cuts and blades disposed of in yellow sharps container. Any unused tissue, media or containment vessel should be decontaminated in 2% virkon.

EQUIPMENT INFORMATION

General points:

Authorisation for use of Class II hood and centrifuge is required.

The Class II cabinets are designed to protect the operator and the work. The escape of airborne particles generated within the cabinet is controlled by means of an inward airflow through the working front aperture, with HEPA filtration of the exhaust air. Use of the hood and good laboratory practice minimises bacterial and fungal contamination of the cultured cells. Aseptic technique is required for culturing and refers to a procedure done under sterile conditions. The hood should be wiped down with either 70% ethanol or 1:100 Trigene before and after work.

The centrifuge utilises centrifugal force to separate on the basis of size and density between the liquid and solid phases. The cellular material pellets at the bottom of the tube with the liquid on top.

Equipment:

BioMat² Class II hood

Sigma 4K15 refrigerated centrifuge

Grant OLS200 shaking waterbath, 37°C

LEEC CO₂ incubator, set at 37°C and 5% CO₂ humid atmosphere. Humidity achieved by tray of distilled water with a teaspoon of copper sulphate/ litre. CO₂ regulator set at 2 psi to deliver 5%.

Motic AE31 inverted microscope to monitor attachment, growth and health of cultured cells.

Information on the above equipment and any service contract held with the supplier can be obtained from the laboratory manager located in Room 1128

MEDIA**Collection**

DMEM
1% FBS
1 ml Primocin/ 500 ml

Prep

DMEM (Sigma)

Culturing

500 ml DMEM F12
10 ml L-glutamine
1 ml Primocin
50 ml FBS (Biosera)
50 µl recombinant human EGF (Sigma)*
* 0.2 mg/ml in 1mg/ml BSA solution in PBS. 0.2 µm filtered. 50 µl aliquots in sterile eppendorfs stored at -20°C

REAGENTS**PBS**

Ca²⁺ / Mg²⁺- free (Sigma)

DNase1

4 mg/ml PBS, 0.2 µm filtered. Store as 100 µl aliquots in sterile eppendorfs at -20°C

MgCl₂

100 mM in H₂O, 0.2 µm filtered. Store at 4°C

Dispase

10 mg/ml PBS, 0.2 µm filtered. Leave on ice for 30 min before filter sterilisation. Store as 500 µl aliquots in sterile eppendorfs at -20°C

Collagenase

20 mg/ml PBS, 0.2 µm filtered. Leave on ice for 30 min before filter sterilisation. Store as 500 µl aliquots in sterile eppendorfs at -20°C

Filter sterilisation is achieved by use of a syringe of appropriate size to which is attached a syringe filter unit of 0.2 µm pore size.

TISSUE CULTURE CONSUMABLES:

All tissue culture consumables come pre-packed and sterile.

Pipette tips and 1.5 ml or 2 ml eppendorf tubes are sterilised by an autoclave. An autoclave is an instrument that sterilises consumables by subjecting them to high pressure saturated steam at 121 °C for 15-20 min. See SOP28. **Authorisation for use of the autoclave is required.**

METHOD

Biopsy material is collected into collection media and can be one of the following:

- a. full thickness; endometrium attached to myometrium
- b. endometrial scraping
- c. pipelle, tube(s) of predominantly endometrial cells

Transfer biopsy material to Petri dish. Ensure tissue remains moist. Add DMEM. For the full thickness endometrial biopsy, scrape the endometria, the shiny layer, with a scalpel blade. Avoid disturbing the junction between endo- and myo- metrium to prevent contamination with muscle cells.

1. Chop the endometrium into small pieces with the scalpel blades to the consistency of fine mince. For pipelle fraction, finely mince.
2. Transfer with pastette into 30 ml universal and rinse Petri dish with DMEM. Centrifuge 500g, 5 min to collect cells.
3. Resuspend cell pellet in 4ml DMEM, add 1X tube collagenase (C2, 20 mg/ml, 500 µl); 1X tube dispase (D, 10 mg/ml, 500 µl); 1X tube DNase 1 (4mg/ml, 100µl) + 50 µl 100mM MgCl₂. Digest 1.5 hrs at 37°C in shaking water bath. Periodically triturate digest with pastette.
4. Filter digest through 40 µm cell strainer into 50 ml tube. Wash well with DMEM, ~30-40 ml. Flow through (FT) = **stromal #**; retentate = **epithelial #**
5. Back-wash retentate (i.e., invert sieve) into 50 ml tube with 30-40 ml DMEM
6. Centrifuge both fractions 500g, 5 min
7. Resuspend **stromal #** in DMEM, 4ml. Add to 4-5ml Ficoll in v-bottom 15 ml clear tube. Centrifuge 400g, 10 min.
8. Remove layer of cells at interface of Ficoll and media into a 30 ml universal tube. RBC will pellet at bottom of tube. A blue tip with a yellow tip attached is useful here.
9. During Ficoll step, resuspend **epithelial #** in 1ml DMEM and add 1 ml 0.25% trypsin (0.125% final). + 1X tube DNase. Incubate 37°C, 20 min and then pipette for 3-5min (Gilson with 1ml blue tip) to liberate single cells.
10. Add 1ml culture media to inactivate trypsin. Majority of fraction should be digested; there may be cell clusters and some partially intact glands.
11. **Stromal #**-wash X1 DMEM in 30 ml universal tube, centrifuge 500g, 5 min and leave on ice.

12. **Epithelial #**-centrifuge as above and then wash X1 DMEM in 30 ml universal tube.
13. Resuspend cell pellet in 1 ml media and plate both fractions in tissue culture T75cm² flasks or 100 mm dishes, depends on cell yield (15 ml media/flask; 10 ml media/dish) and place in incubator 20-30 min. As it is a crude fractionation, the stromal fraction will have epithelial cells and the epithelial fraction will have stromal cells. Fibroblasts/stromal cells will plate readily and quickly while epithelial cells will take longer to plate. Selective adherence will enrich for each population. Monitor adhesion of cells using the inverted microscope. Using a pipette transfer media (non-adherent cells) to a new culture vessel and add fresh media to the adherent cells.
14. Repeat step 14 if necessary.

SOP History

SOP created by AJV

Other useful SOPs associated with 12 Procedure

SOP01 Disinfection protocol
 SOP03 Endometrial biopsy sample reception
 SOP24 Use of Sigma 4K15 centrifuge
 SOP28 Autoclave procedure
 SOP36 Use of HERMLE 2216MK refrigerated microfuge

Appendices -Associated Documents

REAGENTS

DNase 1 #11284932001	Roche Diagnostics
Primocin #ANT-PM-2	Source Bioscience Lifesciences
DMEM/F12HAM (Biowhittaker) #BE12-719F	Lonza
DMEM #D5671-6X500ml	Sigma
Foetal Bovine Serum #S1810, 500ml	Biosera Ltd
Recombinant Human EGF #E9644-.2MG	Sigma
Trypsin-EDTA solution (0.25%) #T4049-100ml	Sigma
L-glutamine #G7513-100ml	Sigma
Dulbecco's PBS #D8537-6x500ml	Sigma
Collagenase #17018-029, 500mg	Invitrogen
Dispase #17105-041, 5g	Invitrogen
MgCl ₂	Fisher
Ficoll	GE life sciences



Standard Operating Procedure 09


 Liverpool Women's 
 NHS Foundation Trust

STANDARD OPERATING PROCEDURE

Number	09	Version & Issue	2.3
Title	ANTIGEN RETRIEVAL		
Author	Jo Drury	Date	November 2010
Approved		Date	
Review Date	November 2013	Last Reviewed	November 2012

BACKGROUND

The mechanisms of formalin-fixation are thought to be due to the formation of cross-linking bonds between tissue proteins, stabilising them to withstand subsequent processing (Mason and O'Leary 1991). Whilst preserving tissue morphology, the formation of cross-linking bonds may, however modify the antigen's epitopes and/or its electrostatic charges, thus producing weak or false negative staining during immunohistochemical detection of certain proteins. Restoring the epitopes enables the antigen to react with the paratope of the antibody. Methods of proteolytic pre-treatment and heat retrieval of tissue antigenicity aim to restore the avidity of the immune reaction.

Proteinase K is a type of proteolytic agent which is isolated from the saprophytic fungus *Tritirachium album*. It possesses a high specific activity which remains stable over a wide range of temperatures and pH values with substantially increased activity at higher temperature. It particularly unmasks antigens of proteins found in the basement membranes including laminin and collagen IV.

There are 2 commonly used heat retrieval buffers, which vary in their pH. Heat causes cross-linked protein epitopes to 'unfold' (in manner similar to DNA denaturation), while buffer solutions aid in maintaining the conformation of the unfolded protein. The citrate based solution is designed to break the protein cross-links, therefore unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies. Tris-EDTA is very useful for low affinity antibodies or when tissue antigens are not intense. This buffer works well for many antibodies, but it often gives high background staining (maybe due to endogenous biotin revealed after this pretreatment), so primary antibody can often be highly diluted.

Standard Operating Procedure 09

HEALTH AND SAFETY REQUIREMENTS

Care needs to be taken when performing heat based retrieval procedures because there is a risk of burns from the hotplate and pressure cooker. There is also a risk of chemical burns from sodium hydroxide and there is a mild irritant effect from citric acid. Suitable personal protection equipment (Nitrile gloves, safety glasses and a lab coat etc) should be worn. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. DO NOT COMMENCE WORK WITH THE PRESSURE COOKER UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND RECEIVED AUTHORISATION FROM LISA HEATHCOTE OR JO DRURY. THE SEAL ON THE PRESSURE COOKER SHOULD BE INSPECTED PRIOR TO EVERY USE AND REPLACED ANNUALLY.

EQUIPMENT INFORMATION:

- Proteinase K supplied by QIAGEN. QIAGEN HOUSE, Fleming Way, Crawley West Sussex, RH10 9NQ
- Diluent
- Humidity Chamber
- Sodium Hydroxide pellets and citric acid are supplied by VWR International Ltd, Poole, BH15 1TD England (Future orders will be obtained from Sigma or Thermo Fisher).
- Trizma base (T1503, Sigma- Aldrich Chemical Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com)
- EDTA (disodium, dehydrate) is supplied by VWR International Ltd, Pole, BH15 1TD. Future orders will be obtained from Sigma (E4884).
- Tefal Clipso Easy 6L pressure cooker and Russell Hobbs hotplate are obtained commercially from John Lewis department store. Annual Insurance check TBC. The seal/gasket needs to be replaced annually. Gasket (part number SA793145) is obtained directly from: <http://www.homeandcook.co.uk> refer to section accessories/pressure cookers. 2010 price £8.50 +£1.50 delivery
- Slide racks are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.
- Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, pH 9.0):

Tris Base	12.1 g
EDTA	3.7 g
Distilled water	2 l to make 5x stock solution

 Mix to dissolve, pH is usually at 9.0. Store this solution at room temperature for 3 months or at 4°C for longer storage.

Standard Operating Procedure 09

METHOD A (Proteinase K):

1. Prepare a volume of proteinase K with a 1:25 concentration of the working stock that is sufficient for 50µl per section. Allow larger volume for larger sections.
2. Once all slides have been de-waxed place into distilled water
3. Prepare humidified chamber
4. Remove slides from the staining dish, wipe the backs of the slides and place onto the humidified chamber.
5. Distribute 50µl of proteinase K onto each section and spread over whole of tissue using a piece of parafilm. Take care whilst spreading to ensure sections do not get dried or damaged in any way.
6. All sections should be incubated with proteinase K for 5 minutes at room temperature.
7. After 5 minutes tap the solution off the slide and place slides in a glass staining dish filled with TBS.
8. See SOP 12 for immunohistochemistry protocol.

METHOD B (Heat based antigen retrieval):

1. Either:
 - (a) Prepare a 10mM solution of citrate buffer, pH=6.0 by adding 3.15g citric acid to 1.5 litre of distilled water and adjust pH to 6.0 with 2M NaOH.Or:
 - (b) Dilute stock 5x Tris-EDTA buffer, pH=9.0 by adding 300 ml to 1.2 l of distilled water.
2. Place buffer in the pressure cooker and place on the hotplate.
3. Turn on the hotplate to the maximum heat setting.
4. Loosely place the lid back on pressure cooker. Bring the buffer to a rolling boil.
5. Immerse the slide rack in the buffer using long forceps.
6. Engage the lid, turn the valve to pressure symbol and press the lid down until it clicks.
7. Set timer for 1 minute - when steam starts to vent, start timer. After 1 minute, turn off the hotplate and release the pressure by turning the vent to the "steam"

Standard Operating Procedure 09

symbol, and cool as quickly as possible by transferring the pressure cooker to the sink and running under cold water.

8. Transfer the slides back to the glass staining jar containing distilled water.
9. Transfer to TBS ensuring that the tissue does not dry out.
10. See SOP 12 for immunohistochemistry protocol.

Other useful SOPs associated with 06 Procedure

Embedding samples using the Shandon Histocentre 3 machine (SOP 05).

Preparation of RM biopsy samples (SOP 03).

Preparation of APES coated slides (SOP 07).

Tissue processing using Shandon processing machine (04).

Immunohistochemistry (SOP12).

SOP History

Original SOP prepared by J Drury and L Heathcote in June 2007.

Updated version was prepared in July 2009 to include changes in both Liverpool Women's Hospital and University of Liverpool logos.

Version 2.1 was prepared by K Palial to include Proteinase K antigen retrieval

Version 2.2 was prepared by J Drury to include Tris-EDTA antigen retrieval and generally update information.

Version 2.3 was prepared to update procedures following relocation of laboratories.

Appendices - Associated Documents

	Document	Location
1	Risk assessment for antigen retrieval	Risk assessment folder in the deputy departmental safety advisors office
2	Physical Hazards Code of Practice	Electronic copies are available on the University of Liverpool health and safety intranet: https://www.liv.ac.uk/intranet/safety/codes_of_practice/physical_hazards.pdf
3		

Review Date

November 2013



Standard Operating Procedure 12


 Liverpool Women's 
 NHS Foundation Trust

STANDARD OPERATING PROCEDURE

Number	12	Version & Issue	2.3
Title	Immunohistochemistry: ImmPRESS (Vector)		
Author	Jo Drury	Date	20.09.11
Approved	Dr D Hapangama	Date	
Review Date	May 2012	Last Reviewed	27.04.11

Background

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues. In order to perform the standard staining procedure, first the tissue section has to be prepared (SOP 04, 05 and 06), deparaffinized and then rehydrated (SOP 08). Antigen retrieval (SOP 09) is then performed to break any protein cross-links, therefore unmasking the antigens and epitopes in the tissue sections. The Vector immPRESS™ reagent kit, HRP is a two-step Immunohistochemical staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequentially non-specific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper counts. Any endogenous peroxidase activity is quenched by incubating the specimen with Peroxidase block. The specimen is incubated with an appropriately characterised and diluted mouse primary antibody, followed by incubation with the labelled polymer. Staining is completed by incubation with 3'3'-Diaminobenzidine (DAB)+ substrate - chromogen which results in a brown-coloured precipitate.

Definition

This SOP will explain how to stain tissue using an unlabelled antibody. Please read entire procedure before staining sections. Perform all antibody and staining incubations in a humid chamber and do not allow sections to dry out. Isotype and system controls should also be run. Carefully time all tests. Do not touch test specimens on slides during the staining procedure.

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Standard Operating Procedure 12

41. Repeat step 40.
42. Blot off excess and incubate 5 min in xylene.
43. Blot off excess and incubate 10 min in xylene.
44. Remove a few slides at a time and apply sufficient mountant to cover the section.
45. Choose an appropriately sized coverslip and apply to the slide.
46. Remove air bubbles by using a cocktail stick or yellow pipette tip to gently press on the coverslip and "chase" bubbles to the edge.
47. Leave to dry in fume hood.

SOP History

Original SOP prepared by L Heathcote 21st June 2007.

Version 2.1 prepared by J Drury 16th July 2008.

Version 2.2 prepared by J Drury 20th September 2010 to update detection system from DAKO EnVision to Vector ImmPRESS.

Version 2.3 prepared by J Drury 27th April 2011 to generalise the SOP.

Other useful SOPs associated with 12 Procedure

Disinfection SOP 01, Transport of biological samples SOP 02 and Tissue Processing SOP 04, Embedding Procedure SOP 05, Cutting sections 06, Preparation of sections for staining SOP 08, Antigen retrieval SOP 09, Autoclave procedure SOP 28.

Appendices -Associated Documents

This will have a list of associated template letters and guidelines relevant to the SOP.

	Document	Location
1	Risk and COSHH assessments	Health and Safety folders in the departmental safety advisors office
2		
3		

Review Date: May 2012

